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13. ABSTRACT (Maximum 200 words)

**The Breast Cancer Training Program (BCTP) in the Eppley Cancer Institute of the University of Nebraska Medical Center offers predoctoral and postdoctoral trainees a comprehensive training environment in breast cancer by supporting, in part, an outstanding breast cancer seminar program, a short course in cancer biology, a breast cancer focus group and by providing stipend support to trainees performing research that is highly relevant to breast cancer. In years one and two of this award we have provided stipends to five predoctoral and postdoctoral trainees. Four of five of the predoctoral trainees have completed their graduate training and have secured postdoctoral positions in outstanding laboratories in research areas directly related to breast cancer. One postdoctoral trainee has moved to another institution and five remain in training. Publications in highly ranked journals are beginning to result from the research of the BCTP trainees. We are currently evaluating a large group of highly qualified applicants for support in year three.**

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## I. Key Accomplishments.

***Task 1.*** **We will develop and maintain a Breast Cancer Training Program for graduate students and postdoctoral fellows that will include the following academic programs (programs and activities were detailed in application).** The Breast Cancer Focus Group continues to meet monthly and the Student/Fellow Research Forum continues to meet weekly during the academic year. The 2002 Short Course in Cancer Biology was held May 13-15 and the internationally recognized visiting faculty for this course is listed in Table 1. The Eppley Institute continues to sponsor an outstanding seminar program with a strong emphasis on breast cancer. Speakers on breast cancer in the 2001-02 academic year are listed in Table 2.

**Table 1. Visiting Faculty for 2002 Short Course in Cancer Biology – Cancer Genetics.**

| Faculty | Institution | Title of Section |
|---|---|---|
| Dr. Gerard Evan | University of California, San Francisco | Constructing and Deconstructing a Cancer Genetically |
| Dr. Donna Albertson | University of California, San Francisco | Microarray Based Comparative Genomic Hybridization to Assess Gene Copy Number |
| Dr. William Kaelin | Harvard University and Howard Hughes Med. Institute | Tumor Suppressor Genes and Gene Based Therapies |
| Dr. William Bennett | City of Hope, Beckman Research Institute | Cancer Epidemiology |

**Table 2. Eppley Institute Seminar Speakers on Breast Cancer, 2001-2002.**

| Date | Speaker | Institution | Topic |
|---|---|---|---|
| September 6, 2001 | Dr. Gilbert Smith | NCI | Mammary stem cells |
| October 11, 2001 | Dr. Wei Zheng | Vanderbilt Univ. | Breast cancer epidemiology |
| November 8, 2001 | Dr. David Soloman | NCI | Cripto, mammary gland morphogen |
| November 15, 2001 | Dr. Dihua Yu | U. Texas, M.D. Anderson | ErbB2 and breast cancer chemoresistance |
| January 17, 2002 | Dr. Larry Brody | NCHGR | BRCA1 |
| February 21, 2002 | Dr. Robert Callahan | NCI | Notch, mammary gland development |
| March 14, 2002 | Dr. Itamar Barash | Volcani Institute, Israel | Stat5, mammary carcinogenesis |
| March 28, 2002 | Dr. Daniel Medina | Baylor College of Medicine | Hormones, aneuploidy and mammary carcinogenesis |
| April 18, 2002 | Dr. Michael Gould | Univ. Wisconsin | Genetics of mammary cancer susceptibility |
| May 9, 2002 | Dr. Priscilla Furth | Georgetown Univ. | Hormonal signaling and mammary carcinogenesis |

***Task 2.*** **We will recruit qualified students and fellows to the Breast Cancer Training Program and, through the laboratories of the training faculty, provide a stimulating, comprehensive and multidisciplinary training experience pertaining directly to breast cancer.** Five predoctoral and six postdoctoral trainees have been supported by this training grant during years 1 and 2 of the four year award period. Each of the three predoctoral trainees supported in year 1 and one of the two predoctoral fellows supported in year 2 have now completed their training in the Eppley Institute and have moved to postdoctoral positions in internationally recognized laboratories. Five of the six postdoctoral trainees supported in years 1 and 2 remain in training. A summary of the research of each fellow is presented below.

**Djuana Harvell, Ph.D.; predoctoral trainee supported in year 1.** Dr. Harvell demonstrated that a 40% restriction of dietary energy consumption inhibits estrogen-induced mammary carcinogenesis in the female ACI rat. This inhibition occurs at a step subsequent to development of focal regions of atypical hyperplasia. Two first author manuscripts were published in the past year (Appendices 1 and 2), and a third has been submitted for publication. Dr. Harvell is now a postdoctoral fellow in the laboratory of Dr. Kate Horwitz at the University of Colorado Health Sciences Center, where she is continuing to study the role of steroid hormones in breast cancer.

**Michelle VanLith, Ph.D.; predoctoral trainee supported in year 1.** Dr. VanLith defined the cellular bases of tumor-specific immune responses to MUC-1. She is first author of a manuscript, listed below, that has been accepted for publication. A second first author manuscript has been submitted for publication. Dr. VanLith is currently a postdoctoral fellow in the laboratory of Dr. V. Englehard at the University of Virginia, working in the area of tumor immunology.

**Jennifer Brennan, Ph.D.; predoctoral trainee supported in year 1.** Dr. Brennan demonstrated that kinase suppressor of ras (KSR) cycles through the nucleus in a phosphorylation dependent manner. Cellular localization was also impacted by specific interactions with MEK. A first author manuscript detailing this study was published in the *Journal of Biological Chemistry* (Appendix 3). Dr. Brennan is currently a postdoctoral fellow at St. Jude Children's Hospital working in the laboratory of Dr. John Cleveland.

**Martin Tochacek, Ph.D.; predoctoral trainee supported in year 2.** Dr. Tochacek mapped several genetic loci that determine susceptibility to estrogen-induced mammary cancer in crosses between the highly susceptible ACI strain and two different resistant rat strains. Dr. Tochacek contributed to one published manuscript (Appendix 4) and two first author manuscripts have been submitted for publication. Dr. Tochacek is currently a postdoctoral fellow at Duke University, working in the laboratory of Dr. Donald McDonnell, studying steroid hormones action and breast cancer.

**Kimberly Wielgus; predoctoral trainee supported in year 2.** Ms. Wielgus is working toward the Ph.D. in nursing and is investigating fatigue in patients with advanced stage breast cancer. Although Ms. Wielgus is in the early stages of her dissertation research, her participation in the activities of the Breast Cancer Research Program has enabled her to gain a fundamental understanding of the disease process as well as its genetic and molecular bases. Ms. Wielgus competed successfully for a four-year Scholarship in Cancer Nursing from the American Cancer Society.

**Benjamin Xie, M.D., Ph.D.; postdoctoral trainee supported in year 1.** Dr. Xie demonstrated that expression of progesterone receptor (PR) is much higher in the focal regions of atypical hyperplasia and mammary carcinoma induced in ACI rats by continuous treatment with estradiol than in normal or hyperplastic mammary glands. These data are included in two published manuscripts. Dr. Xie also demonstrated that expression of Cdkn2a is markedly down-regulated as an early event in estrogen-induced mammary carcinogenesis. A manuscript describing these data is in preparation.

**Constance Dooley, Ph.D.; postdoctoral trainee supported in year 1.** Dr. Dooley tested the hypothesize that ectopic kinase suppressor of ras (KSR) will inhibit the transformation properties of human cancer cells in vitro and the tumorigenic potential of mammary tissue in vivo. Dr. Dooley successfully generated high-titer recombinant baculovirus for full-length KSR, KSR with two mutated phosphorylation sites, the carboxy terminal half of KSR, the amino terminal half of KSR, and two forms of KSR with reduced or absent activity. A manuscript describing these studies is in preparation. Dr. Dooley recently moved to a new postdoctoral training position at the University of Utah.

**David Smith, Ph.D.; postdoctoral trainee supported in year 1.** Dr. Smith investigated the regulation of the human *MUC1* gene. *MUC1* has been shown to be up-regulated in many forms of cancer including breast. He performed *in vivo* footprinting experiments to locate the positions of transcription factor binding sites in the promoter region of the *MUC1* gene. Finally, he initiated a translational study in which cDNA array technologies are being used to compare gene expression profiles in primary breast cancers and associated axillary lymph node metastasis.

**Beverly Schaffer, Ph.D.; postdoctoral trainee supported in year 2.** Dr. Schaffer joined the BCTP in December of 2001. She is generating congenic rat lines in which Brown Norway alleles for *Emca1*, *Emca2* and *Emca3* are carried on the ACI background. We have demonstrated that these *Emca* loci determine susceptibility to estrogen-induced mammary cancer in crosses between the ACI and BN rat strains. The congenic lines will be characterized to define the roles of each *Emca* locus in estrogen-induced mammary carcinogenesis and to fine map each *Emca* locus.

**Nicholas Moniaux, Ph.D.; postdoctoral trainee supported in year 2.** Dr. Moniaux has cloned and characterized the MUC4 genes from rat and human and is defining the interactions between MUC4 and HER2. He is testing the hypothesis that MUC4/HER2 interactions contribute to pathogenesis of breast cancer.

**Adrian Reber, Ph.D.; postdoctoral trainee supported in year 2.** Dr. Reber is investigating the role of invariant chain protein (Ii) on MHC class I molecules in different breast cancer cell lines from humans, rats and mice. It has been demonstrated that Ii binds only to folded, peptide free, class I molecules and results in increased cell surface expression of class I. Experiments underway will test the hypothesis that Ii may be used to increase anti tumor immune responses in rat and mouse mammary cancer models.

*Task 3.* **We will maintain oversight of the Breast Cancer Training Program to ensure that all progress reports and communications are submitted as required and that the training faculty and trainees fulfill their respective obligations to the program.** We are currently evaluating predoctoral and postdoctoral candidates for support from the training grant. All progress reports have been submitted as required. All activities associated with the Breast Cancer Training Program, as described in our application, are being organized.

## II. Reportable Outcomes.

### A. Published Manuscripts (funded trainees are underlined):

Brennan, J.A., Volle, D.J., Chaika, O.V. and Lewis, R.E. Phosphorylation regulates the nucleocytoplasmic distribution of kinase suppressor of ras. J. Biol. Chem. 277:5369-5377, 2002.

Harvell, D.M.E., Strecker, T.E., Xie, B., Buckles, L.K., Tochacek, M., McComb, R.D. and Shull, J.D. Diet-gene interactions in estrogen-induced mammary carcinogenesis in the ACI rat. J. Nutrition 131:3087S-3091S, 2001.

Harvell, D.M.E., Strecker, T.E., Xie, B., Pennington, K.L., McComb, R.D. and Shull, J.D. Dietary energy restriction inhibits estrogen-induced mammary, but not pituitary, tumorigenesis in the ACI rat. Carcinogenesis 23:161-169, 2002.

Moniaux, N., Escande, F., Porchet, N., Aubert, J.P. and Batra, S.K. Structural organization and classification of the human mucin genes. Front. Bioscience 6:D1192-1206, 2001.

Shiraga, T., Smith, D., Nuthall, H.N., Hollingsworth, M.A. and Harris, A. Identification of two novel elements involved in human MUC1 gene expression in vivo. Mol. Med. 8:33-41, 2002.

Shull, J.D., Pennington, K.L., Reindl, T.M., Snyder, M.C., Strecker, T.E., Spady, T.J., Tochacek, M. and McComb, R.D. Susceptibility to estrogen-induced mammary cancer segregates as an incompletely dominant phenotype in reciprocal crosses between the ACI and Copenhagen rat strains. Endocrinology 142:5124-5130, 2001.

VanLith, M.L., Kohlgraf, K.G., Sivinski, C.L., Tempero, R., and Hollingsworth, M.A. MUC1-Specific Anti-Tumor Responses: Molecular Requirements for CD4 mediated responses. International Immunology In Press, 2002.

**B. Degrees obtained:**

| | |
|---|---|
| Michelle VanLith, Ph.D. | degree granted August 2001. |
| Djuana Harvell, Ph.D. | Degree granted December 2001. |
| Jennifer Brennan, Ph.D. | degree granted December 2001. |
| Martin Tochacek, Ph.D. | degree granted June 2002. |

**C. Cell lines generated:**

| | |
|---|---|
| B. Xie/J. Shull | normal mammary epithelium, ACI rat |
| B. Xie/J. Shull | normal mammary epithelium, Copenhagen rat |
| B. Xie/J. Shull | estrogen-induced mammary carcinoma, ACI rat |

**D. Related funding:**

Kim Wielgus, Scholarship in Cancer Nursing, American Cancer Society, 2002-2006.

**E. Employment received:**

Dr. Michelle VanLith (Ph.D., June 2001) accepted a postdoctoral position in the laboratory of Dr. V. Englehard at the University of Virginia, working in the area of tumor immunology.

Djuana Harvell (Ph.D., December 2001) accepted a postdoctoral position in the laboratory of Dr. Kate Horwitz at the University of Colorado Health Science Center. She is studying steroid hormones and breast cancer.

Jennifer Brennan (Ph.D., December 2001) accepted a postdoctoral position in the laboratory of Dr. John Cleveland at St. Jude Children's Hospital.

Martin Tochacek (Ph.D., June 2002) accepted a postdoctoral position in the laboratory of Dr. Donald McDonnell at Duke University, working in the area of steroid hormone action.

Constance Dooley (postdoctoral trainee, 2000-2002) accepted a postdoctoral position in the laboratory of Monica Vetter at the University of Utah.

Carcinogenesis vol.23 no.1 pp.161–169, 2002

# Dietary energy restriction inhibits estrogen-induced mammary, but not pituitary, tumorigenesis in the ACI rat

Djuana M.E.Harvell[1,2], Tracy E.Strecker[1,3], Benjamin Xie[1], Karen L.Pennington[1], Rodney D.McComb[2] and James D.Shull[1–4]

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**Because of the suggested role of energy consumption and the well-documented role of estrogens in the etiology of breast cancer, we have examined the effect of a 40% restriction of dietary energy consumption on the ability of administered 17β-estradiol (E2) to induce mammary tumorigenesis in female ACI rats. Experiments herein test the hypothesis that at least part of the inhibitory effect of energy restriction on mammary tumorigenesis is exerted downstream of potential effects of dietary manipulation on the production of estrogens by the ovaries. Ovary-intact ACI rats were fed a control or a 40% energy-restricted diet and were either treated continuously with E2 from subcutaneous Silastic tubing implants or received no hormone treatment. Mammary cancers rapidly developed in E2-treated rats fed the control diet; within 216 days of initiation of E2 treatment 100% of the population at risk exhibited palpable mammary tumors. Dietary energy restriction markedly inhibited E2-induced mammary tumorigenesis, as evidenced by significant reductions in cancer incidence and tumor burden as well as a significant increase in the latency to the appearance of the first palpable cancer. The inhibitory actions of dietary energy restriction on E2-induced mammary tumorigenesis were associated with an inhibition of E2-stimulated mammary cell proliferation. However, this inhibition was insufficient to block induction of lobuloalveolar hyperplasia or appearance of focal regions of atypical epithelial hyperplasia. These data suggest that dietary energy restriction inhibits E2-induced mammary cancer by attenuating or retarding the progression of atypical hyperplasia to carcinoma. Expression of progesterone receptor (PR) was up-regulated within the focal regions of atypical hyperplasia and the carcinomas induced by E2, regardless of whether the rats were fed the control or energy-restricted diet. However, circulating progesterone was reduced by dietary energy restriction, suggesting a possible mechanism for inhibition of mammary tumorigenesis. Dietary energy restriction did not inhibit the ability of administered E2 to induce prolactin (PRL)-producing pituitary tumors and associated hyperprolactinemia, indicating that the inhibitory effects of dietary energy restriction on mammary tumorigenesis are tissue specific and independent of circulating E2 and PRL.**

**Abbreviations:** ANOVA, analysis of variance; BrdU, 5-bromo-2′-deoxyuridine; COP, Copenhagen; E2, 17β-estradiol; DMBA, dimethylbenz[*a*]anthracene; MNU, *N*-methylnitrosourea; PRL, prolactin; PR, progesterone receptor; SEM, standard error of the mean; SD, standard deviation.

## Introduction

Several dietary factors, including dietary energy consumption, have been implicated as determinants of breast cancer risk in human populations (1). For example, both prospective and case-control studies associate height and/or body mass index with breast cancer risk and provide indirect evidence that energy consumption and/or balance influence the development of breast cancer (2,3). Supporting these epidemiologic data are numerous studies demonstrating that dietary energy restriction markedly inhibits mammary tumorigenesis in a variety of rodent models (4–8). In several laboratory studies, the inhibition of mammary tumorigenesis by energy restriction has been associated with reductions in mammary epithelial cell proliferation or circulating levels of estrogens and/or prolactin (PRL) (8–17). Because these ovarian and pituitary hormones regulate mammary gland growth, differentiation and function, it has been hypothesized that reduction of hormone output by the ovary and/or pituitary is one important mechanism through which the inhibitory effects of energy restriction on mammary tumorigenesis are manifested (16–18).

Abundant data from epidemiologic studies demonstrate that ovarian hormones, particularly estrogens, play an important role in the etiology of breast cancer (19–21). Moreover, prophylactic treatment with the anti-estrogen tamoxifen reduced by ~50% the incidence of breast cancer in a population of women at high risk of this disease (22). It has been hypothesized that estrogens contribute to breast cancer etiology, at least in part, by increasing the rate of mammary cell proliferation, thereby promoting the accumulation of somatic mutations (23). Cell proliferation in the human breast epithelium is highest during the luteal phase of the menstrual cycle, when circulating progesterone is highest (24). Together, the data suggest that both estrogens and progestins contribute to breast cancer etiology.

Recent studies from our laboratory reveal the female ACI rat to be a unique and physiologically relevant animal model for the study of breast cancer. Ovary-intact ACI rats rapidly develop mammary cancers when the level of 17β-estradiol (E2) in the systemic circulation is maintained in the high physiologic range by release of E2 from subcutaneous implants, whereas mammary cancers very rarely develop in this rat strain in the absence of exogenous E2 (25). Continuous stimulation of the mammary gland by E2 results in induction of lobuloalveolar hyperplasia, subsequent appearance of focal regions of atypical epithelial hyperplasia, and ultimately, development of multiple, independently arising, mammary cancers (25,26). Ovariectomy markedly inhibits development of E2-induced mammary cancers, and the epithelial cells within both the focal regions of atypical hyperplasia and the mammary carcinomas exhibit increased expression of progesterone recep-

tor (PR) relative to the surrounding epithelium (25,26). These data suggest that progesterone and its receptor may contribute to the development of E2-induced mammary cancers, and that atypical hyperplasia may be a precursor lesion to carcinoma. In contrast to the high degree of susceptibility of the ACI rat strain to E2-induced mammary cancers, the genetically related Copenhagen (COP) rat strain is much less susceptible to E2-induced mammary tumorigenesis (27,28). Interestingly, the mammary epithelia of the female ACI rat exhibits a greater proliferative response to E2 than does that of the female COP rat, suggesting a possible mechanism for the observed differences in the susceptibilities of these two strains to E2-induced mammary cancers (26). In a genetic cross between the susceptible ACI rat and the resistant COP rat, susceptibility to E2-induced mammary cancers is inherited as an incompletely dominant phenotype (29). Thus, the ACI rat provides a genetically defined and physiologically relevant model in which to study diet/hormone interactions in mammary cancer development.

Because of the suggested role of energy consumption and the well-documented role of estrogens in the etiology of breast cancer, we have examined the effect of a 40% restriction of dietary energy consumption on E2-induced mammary tumorigenesis in female ACI rats. To our knowledge, this is the first study to examine the action of dietary energy restriction in an animal model where mammary cancer is induced solely through the actions of a naturally occurring hormone. Because exogenous E2 is the inducing agent in this model, this study allowed us to test the hypothesis that at least part of the inhibitory effect of energy restriction on mammary tumorigenesis is downstream of potential effects of energy restriction on output of estrogens by the ovaries. Moreover, because the administered E2 maintains production of pituitary derived PRL at a high level (30,31), this study also allowed us to examine the ability of dietary energy restriction to inhibit mammary tumorigenesis independent of any inhibitory effect on pituitary PRL output. The data presented herein demonstrate that dietary energy restriction markedly inhibited development of E2-induced mammary cancers in the ACI rat, apparently by attenuating or retarding the progression of pre-neoplastic lesions to carcinoma.

## Materials and methods

*Care and treatment of animals*

All protocols involving live animals were approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center. Female ACI rats were obtained from Harlan (Indianapolis, IN) and were housed one animal per cage within a barrier animal facility with controlled temperature, humidity and lighting cycle (12 h light/12 h dark). The rats were initially fed a semi-purified control diet that was formulated in accordance with guidelines established by the American Society of Nutritional Science (32). One week later, the rats were randomly assigned to groups fed either this control diet or an energy-restricted diet. The compositions of these diets are illustrated in Table I, and the methods used in their preparation have been described previously (30,33,34) . The rats fed the control diet were allowed to eat *ad libitum*, and the amount of food consumed by these animals was monitored weekly. The rats fed the energy-restricted diet were fed each day, at the beginning of the dark phase of the lighting cycle, 0.64 g of food per g of food consumed per day by the rats fed the control diet. Consequently, the animals fed the energy-restricted diet consumed 40% less energy, derived from carbohydrate and fat, but equivalent amounts of protein, vitamins, minerals, fiber and other nutrients, relative to that consumed by animals fed the control diet. The rats were allowed continuous access to tap water. Body weights were monitored weekly.

Silastic tubing implants, either empty or containing 27.5 mg of crystalline E2, were prepared as described previously (25,27). When the animals were 59 days of age, the implants were surgically inserted subcutaneously in the interscapular region while the rats were under ether anesthesia. The animals ($n$ = 21 per experimental group) were examined twice weekly for the presence of palpable mammary tumors. The location and approximate size of each mammary tumor were recorded at each examination. A rat was killed when its largest mammary tumor approached 2.0 cm in diameter or if signs of morbidity were observed. A second group of animals ($n$ = 8 per experimental group) was killed following 84 days of E2 treatment, to allow the interactions between dietary energy consumption and E2 treatment to be examined at a defined time point. Four hours prior to killing, each rat received an intraperitoneal injection of 5-bromo-2′deoxyuridine (Sigma Chemical Co., St Louis, MO) solubilized in sterile phosphate-buffered saline and administered at a dose of 50 mg/kg body weight.

**Table I.** Formulation of experimental diets

| Diet component[a] | Control diet | EnRes diet |
|---|---|---|
| Corn oil | 5.0 | 3.8 |
| Casein | 20.0 | 31.1 |
| DL-methionine | 0.3 | 0.5 |
| Glucose | 15.0 | 11.4 |
| Dextrin | 49.9 | 38.1 |
| Fiber[b] | 5.0 | 7.8 |
| AIN mineral mix | 3.5 | 5.4 |
| AIN vitamin mix | 1.0 | 1.5 |
| Choline bitartrate | 0.3 | 0.4 |
| Grams fed per grams consumed by control animals | 1.0 | 0.64 |

[a]Ingredients were obtained from Harlan-Teklad or Sigma Chemical Co. Grams of each component per 100 g of diet are indicated. AIN, American Institute of Nutrition.
[b]Non-nutritive fiber (purified wood cellulose, 74–75% crude fiber).

*Collection and evaluation of mammary tissues*

The rats were killed by decapitation. The pituitary glands were removed and weighed. Trunk blood was collected, allowed to clot at 4°C and centrifuged. The serum was collected and stored at –80°C. The levels of E2 (25), PRL (26) and progesterone (35) in serum from trunk blood were measured by radioimmunoassay. The location and size of each mammary tumor were recorded at necropsy. The diameter for each tumor was determined in two perpendicular dimensions with calipers, and the tumor volume was calculated using the formula $V = 4/3\pi r^3$ where $r$ is half the average diameter. Mammary tissues, both grossly normal and tumors were collected, placed in Lillie's fixative for 24 h, processed and embedded in paraffin. Sections from each tissue were stained with hematoxylin and eosin and evaluated by light microscopy. The fraction of mammary epithelial cells in the S phase of the cell cycle was quantified by immunohistochemical identification of cells that incorporated BrdU as described previously (26,27,30,33). At least 2000 mammary cells per rat were scored for incorporation of BrdU. Mammary cells expressing PR were identified immunohistochemically using a polyclonal antibody (C19; Santa Cruz Biotechnology, Santa Cruz, CA) that was diluted 1:100 in 10% normal goal serum (26). An inguinal mammary gland from each animal was collected, prepared as a whole mount and stained with alum carmine for whole mount analysis (Sigma) (26).

*Statistical analysis of data*

Data are presented as the mean ± SEM or mean ± SD. Differences between means were assessed using Student's $t$-test and/or two-way ANOVA with Newman–Keul's post hoc test. Mammary tumor latency and incidence were analyzed by the log rank test for comparison of cumulative incidence curves and the Fischer's exact test, respectively. $P$ values $<0.05$ were considered statistically significant.

## Results

*Effects of estrogen treatment on food consumption and growth*

Untreated female ACI rats fed the control diet consumed an average of 10.3 g of diet/day throughout the course of the experiment (Figure 1A). Food consumption was increased by 8%, to 11.2 g/day ($P < 0.05$), in rats treated with E2. When fed the control diet, both untreated and E2-treated rats grew rapidly until ~84 days of age (Figure 1B). Thereafter, both the

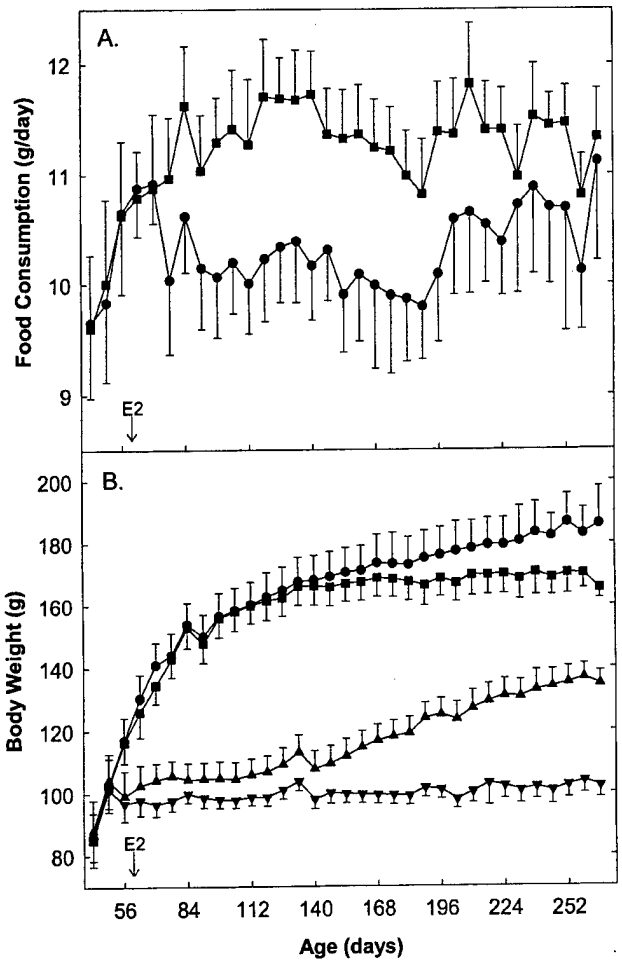


**Fig. 1.** Effects of E2 treatment and diet on food consumption and growth. Female ACI rats were obtained at 42 days of age, allowed unlimited access to the control diet for 7 days, and then assigned at random to either the control diet or the energy-restricted diet. Food consumption by animals fed the control diet was monitored weekly. A 40% restriction of energy consumption was imposed by feeding 0.64 g of the energy-restricted diet per g consumed by rats fed the control diet. Treatment with E2 was initiated when the animals were 59 days of age. **(A)** Each data point represents the average amount of diet consumed per rat per day ± SD. **(B)** Each data point represents the average body weight±SD. Symbols: filled circle, control diet untreated; filled square, control diet, E2 treated; filled triangle, 40% energy restricted, untreated; filled inverted triangle, 40% energy restricted, E2 treated.

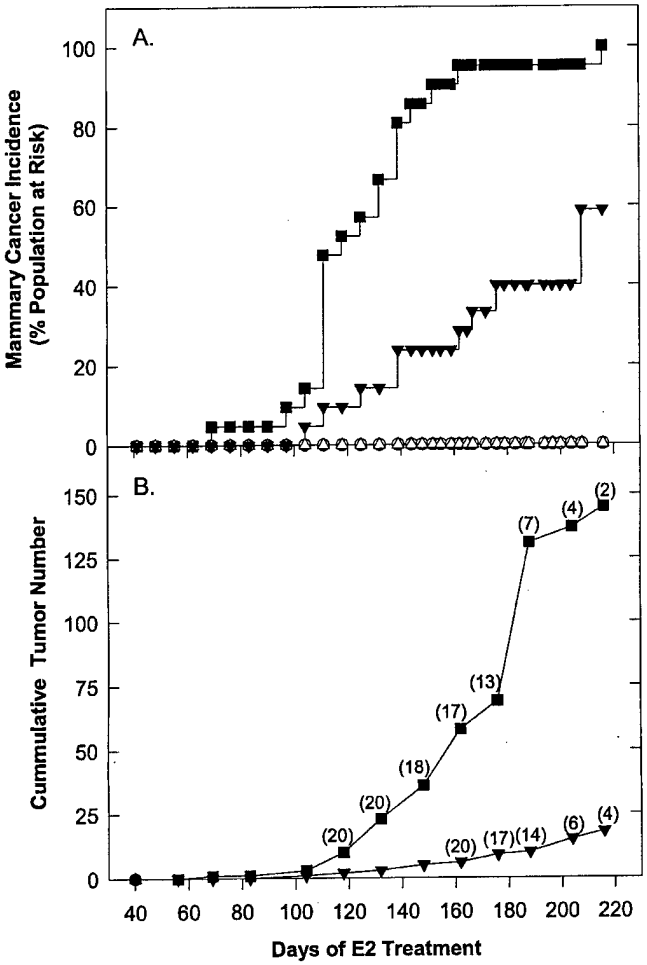


**Fig. 2.** Dietary energy restriction inhibits E2-induced mammary tumorigenesis in female ACI rats. Animals were treated as described in Figure 1 and the Materials and methods. **(A)** Each data point represents the percentage of rats at risk exhibiting palpable mammary tumors at the indicated times relative to initiation of E2 treatment. **(B)** Each data point represents the cumulative number of palpable mammary tumors observed in the E2-treated groups. The numerals in parentheses indicate the number of animals in each experimental group remaining alive at the specified time point; this number equals 21 if not indicated. Symbols: open circle, control diet, untreated; filled square, control diet, E2 treated; open triangle, energy-restricted diet, untreated; filled inverted triangle, energy restricted diet, E2 treated.

untreated and E2-treated rats continued to grow at a reduced rate until ~150 days of age, when growth in the E2-treated rats virtually ceased and the average body weight in the untreated population began to diverge from that of the treated population. At 231 days of age, the untreated animals weighed significantly more ($P < 0.05$) than the E2-treated animals. Together, the data indicate that E2 exerts an inhibitory effect on growth that is independent of food consumption. A 40% restriction of dietary energy consumption markedly inhibited growth. Untreated animals fed the energy-restricted diet grew at a slow, but steady, rate over the course of the experiment. At completion of the study, the energy-restricted animals weighed 28% ($P < 0.05$) less than the rats fed the control diet. E2-treated rats fed the energy-restricted diet maintained a constant body weight throughout the course of the experiment. At completion of the study, the energy-restricted, E2-treated, animals weighed 38% ($P < 0.05$) less than the E2-treated rats fed the control diet.

*Dietary energy restriction markedly inhibits estrogen-induced mammary tumorigenesis*

Administration of E2 to female ACI rats fed the control diet resulted in the rapid development of mammary tumors (Figure 2A). In this population, the first palpable mammary tumor was detected 69 days following the initiation of E2 treatment, and 100% of the treated population exhibited one or more palpable mammary tumors within 216 days of treatment. The median and mean latencies to the appearance of the first palpable mammary tumor in E2-treated rats fed the control diet were 118 and 126 days, respectively. A 40% restriction of dietary energy consumption markedly inhibited development of E2-induced mammary tumors. In the energy-restricted E2-treated population, the first palpable mammary tumor was observed

following 104 days of treatment, and 59% (10/17) of the population at risk had tumors by 207 days. The median and mean latencies in the energy-restricted rats bearing E2-induced mammary tumors were 150 and 154 days, respectively. Both median latency and final mammary tumor incidence differed significantly between the groups of E2-treated animals fed the control diet versus the energy-restricted diet ($P < 0.001$). Five of 21 rats in the energy-restricted, E2-treated group exhibited morbidity, apparently due to an E2-induced pituitary tumor, and were killed following 176, 188 (two rats), 195 and 201 days of E2 treatment. Four of these animals were free of palpable mammary tumors at the time of death. Untreated, ovary-intact, ACI rats fed either the control or energy-restricted diet did not develop mammary tumors over the course of this experiment (Figure 2A).

In addition to reducing mammary tumor incidence and increasing latency, consumption of an energy-restricted diet significantly reduced mammary tumor burden. A total of 145 mammary tumors were induced in the population of 21 ACI rats fed the control diet and treated with E2 for 177 ± 26 days, a yield of 6.9 tumors/rat at the time of death (Figure 2B). In contrast, only 18 mammary tumors were induced in the 21 ACI rats fed the energy-restricted diet and treated with E2 for 193 ± 19 days, a yield of 0.9 tumors/rat. The total volume of mammary tumor tissue, defined as the sum of the volumes of all the mammary tumors observed per rat at necropsy, averaged 5584 $mm^3$ in the population of E2-treated rats fed the control diet, compared with 458 $mm^3$ in the population of E2-treated rats fed the energy-restricted diet (data not shown). The differences in tumor number and volume observed in the E2-treated animals fed the control or energy-restricted diets were both statistically significant ($P < 0.01$).

*Dietary energy restriction inhibits estrogen-induced cell proliferation in normal, but not neoplastic, mammary tissues*

Cell proliferation within the mammary epithelium was examined following 84 days of E2 treatment, a time preceding the appearance of the vast majority of induced mammary tumors (Figure 3A), and following 180–210 days of E2 treatment, a range of time points when animals were being killed due to the presence of mammary tumors (Figure 3B). A marked stimulatory effect of E2 on mammary epithelial cell proliferation in rats fed the control diet was evident at both time points; the fraction of cells staining positive for BrdU was increased from ~0.5% in untreated rats to 3.5–4.0% in E2-treated rats. The ability of E2 to induce mammary cell proliferation was partially, but significantly ($P < 0.01$), attenuated in animals fed the energy-restricted diet. In these animals, the fraction of cells staining positive for BrdU was ~2% at both time points. Dietary energy restriction did not consistently affect mammary cell proliferation in untreated rats. Although dietary energy restriction attenuated the ability of E2 to stimulate cell proliferation within the hyperplastic mammary epithelium, cell proliferation in E2-induced mammary tumors was not inhibited by energy restriction. Approximately 7% of cells in E2-induced mammary tumors incorporated BrdU regardless of whether the rats consumed the control or the energy-restricted diet (Figure 3C). This level of cell proliferation was significantly greater ($P < 0.01$) than in the surrounding hyperplastic mammary epithelium.

*Effects of dietary energy restriction and estrogen on mammary gland morphogenesis and expression of PR*

Examination of mammary gland whole mounts revealed that the glands of untreated female ACI rats fed the control diet

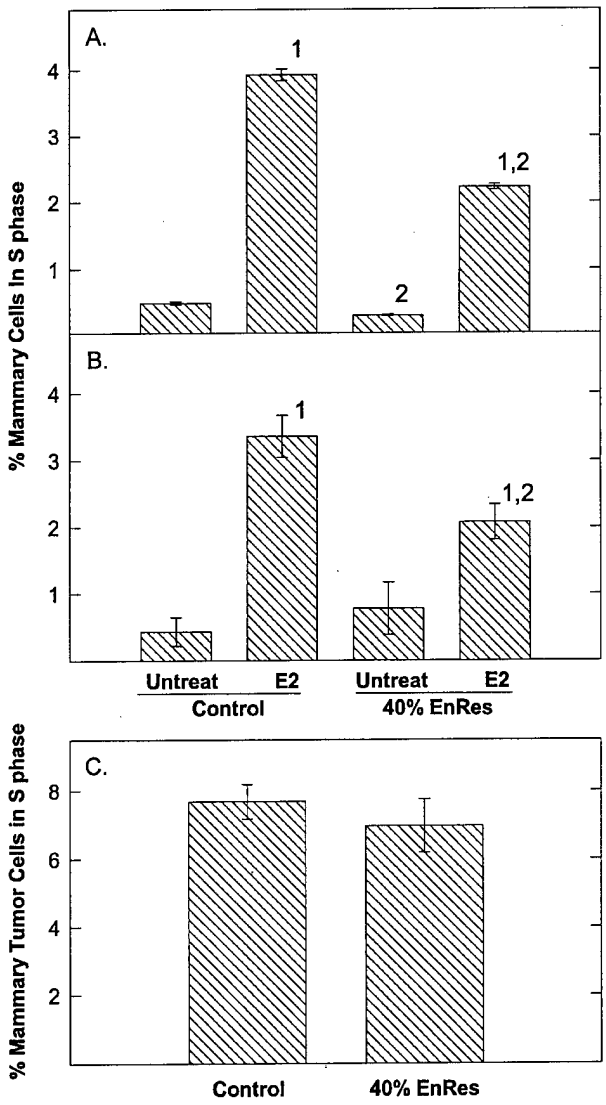


**Fig. 3.** Dietary energy restriction inhibits E2-induced cell proliferation in the mammary gland of ACI rats, but not in mammary cancers. Mammary cells in the S phase of the cell cycle were identified by immunohistochemical detection of BrdU. Each data bar represents the mean (± SEM, $n$ = 6–8 animals chosen at random) number of mammary cells incorporating BrdU, expressed as a percentage of total mammary cells. Cell proliferation was examined in normal mammary tissues following 84 days (**A**) and 180–210 days (**B**) of E2 treatment, and in mammary cancers (**C**) induced by 180–210 days of E2 treatment. Numerals: 1, indicates a statistically significant difference ($P < 0.05$) between untreated and E2-treated rats fed the same diet; 2, indicates a statistically significant difference ($P < 0.05$) between similarly treated animals fed the different diets.

were comprised of ducts exhibiting higher order branching as well as numerous lateral and alveolar buds (Figure 4A). In contrast, the mammary glands of rats fed the energy-restricted diet exhibited a lesser degree of ductal branching and bud development (Figure 4B). Representative ducts for untreated rats fed the control and the energy-restricted diet are illustrated (Figure 4C and D). The interlobular stroma of rats fed the control diet (Figure 4C) was comprised of adipocytes that were generally more uniform in size and larger than were the stromal adipocytes from animals fed the energy-restricted diet

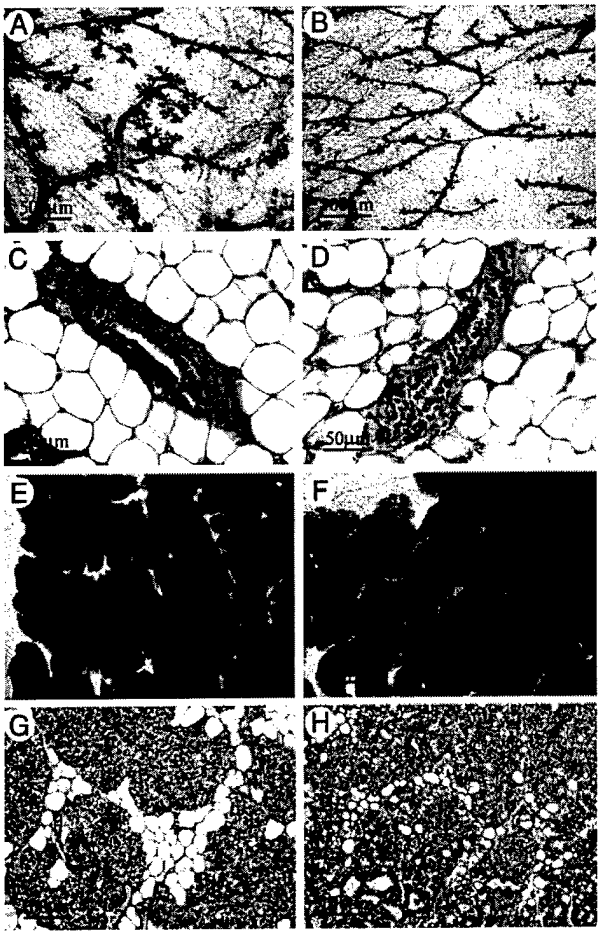


**Fig. 4.** Effects of E2 and dietary energy restriction on mammary gland development. Mammary tissues were evaluated by microscopic examination of mammary gland whole mounts (A, B, E, F) and thin sections stained with hematoxylin and eosin (C, D, G, H). Relative to mammary glands from untreated female ACI rats fed the control diet (**A**), glands from untreated rats fed the energy-restricted diet (**B**) exhibited fewer branches and buds. Interlobular adipocytes were larger and more uniform in size in female rats fed the control diet (**C**), when compared with the adipocytes of untreated, energy-restricted rats (**D**). ACI rats fed either the control (**E** and **G**) or energy-restricted (**F** and **H**) diet and treated continuously with E2 for 84 days exhibited marked lobuloalveolar hyperplasia. Stromal adipocytes were larger in E2-treated rats fed the control diet (G) relative to rats fed the energy-restricted diet (H).

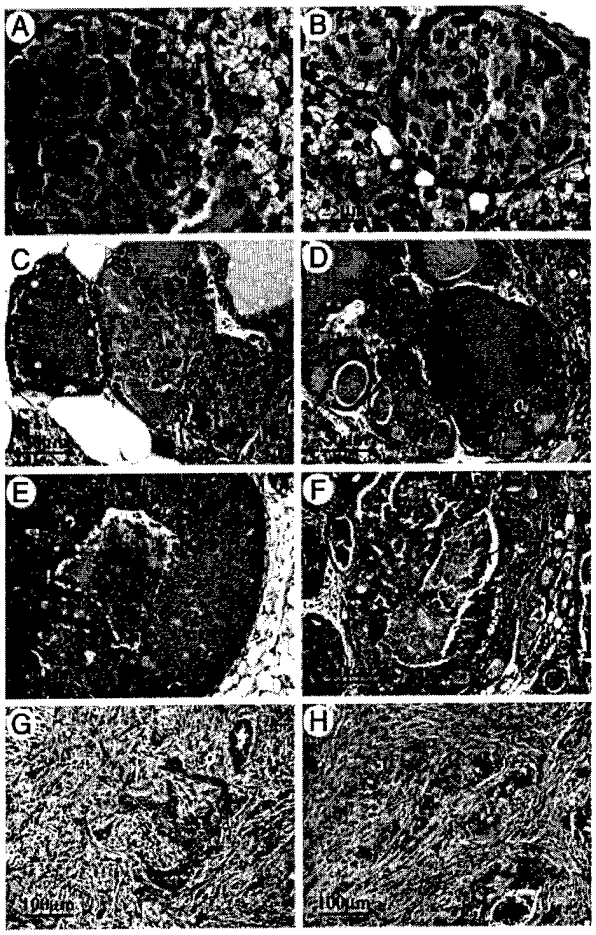


**Fig. 5.** Histologic features of focal regions of atypical hyperplasia, intraductal carcinoma and invasive carcinoma induced in female ACI rats by continuous treatment with E2. Focal regions of atypical epithelial hyperplasia were observed in female ACI rats fed either the control (**A**) or the energy-restricted diet (**B**) and treated with E2 for 84 days. Many of the atypical hyperplastic lesions from rats fed either the control (**C**) or the energy-restricted (**D**) diets and treated with E2 beyond the 84 day time point, exhibited dilated lumens. Although intraductal carcinomas of the comedo type comprised the majority of the E2-induced mammary tumors in rats fed either experimental diet [(**E**), control diet; (**F**), energy-restricted diet], invasive carcinomas exhibiting desmoplastic reaction [(**G**), control diet; (**H**) energy-restricted diet] were also observed.

(Figure 4D). Examination of mammary gland whole mounts (Figure 4E and F) and stained sections (Figure 4G and H) revealed that treatment with E2 for 84 days induced marked lobuloalveolar hyperplasia in female ACI rats fed either the control or energy-restricted diet. Interlobular stromal adipocytes were generally larger in E2-treated rats fed the control diet relative to E2-treated rats fed the energy-restricted diet (Figure 4G and H). Similar results were observed in animals examined at later time points.

Focal regions of atypical epithelial hyperplasia were observed in the mammary glands of female ACI rats fed either the control or energy-restricted diet and treated with E2 for 84 days (Figure 5A–D). The number of these lesions increased as the duration of E2 treatment was extended beyond this time point (data not shown). These focal regions of atypical hyperplasia were characterized by an expanded acinus comprised of cells exhibiting slightly enlarged nuclei and dense cytoplasmic staining resulting from a decrease in vacuolation (Figure 5A and B). In addition, many of the atypical hyperplastic foci were further characterized by the presence of secreta within a dilated lumen (Figure 5C and D). Lesions of this latter type were common in the mammary glands of E2-treated rats fed either the control or the energy-restricted diet. Histologic examination revealed that 60–80% of the tumors that developed in E2-treated animals fed the control or the energy-restricted diet were carcinomas of the comedo type (Figure 5E and F), whereas 20% were cribriform carcinomas. Papillary carcinomas comprised 20% of the tumors induced in animals fed the control diet, but were not observed among the 18 tumors induced in animals fed the energy-restricted diet. Carcinomas exhibiting invasive features were observed in animals fed either the control or the energy-restricted diet (Figure 5G and H).

Intense PR immunoreactivity was observed within the nuclei of a subset of ductal epithelial cells from untreated female

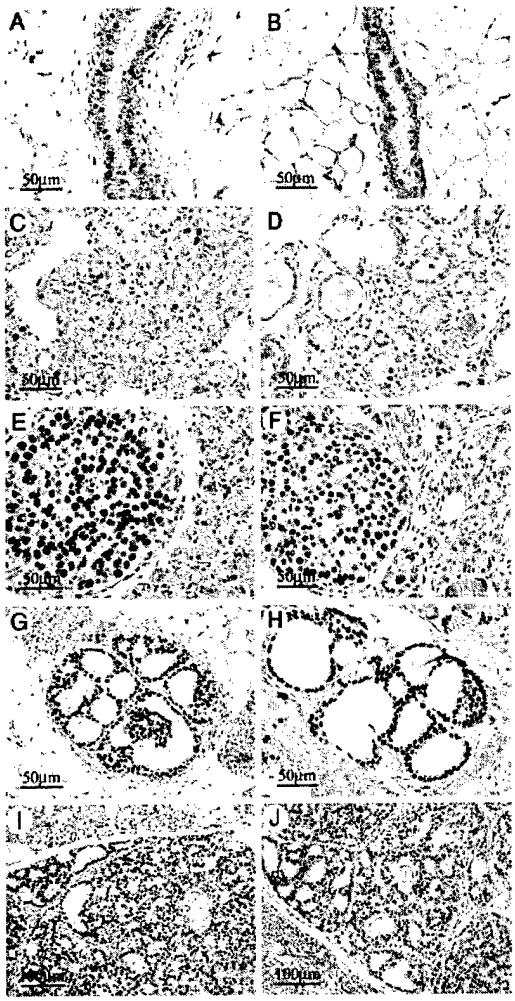


**Fig. 6.** Effects of E2 and dietary energy restriction on expression of PR in normal, hyperplastic and neoplastic mammary tissues. Cells expressing PR were identified by immunohistochemistry. (A, C, E, G and I) PR expression in rats fed the control diet and (B, D, F, H and J) PR expression in rats fed the energy-restricted diet. PR was expressed by a subset of mammary epithelial cells from untreated ACI rats (age matched to E2-treated animals illustrated in C–J), and immunostaining appeared more robust in tissues from untreated rats fed the control diet (**A**), relative to untreated rats fed the energy-restricted diet (**B**). PR was also expressed by a subset of epithelial cells from the hyperplastic mammary tissues of E2-treated rats (**C, D**), and this expression was not affected by diet. The great majority of cells within the focal regions of atypical hyperplasia (**E–H**) and intraductal carcinomas (**I** and **J**) induced by E2 exhibited increased PR expression, relative to the surrounding epithelium. Dietary energy restriction did not detectably affect PR expression in the atypical hyperplasias or carcinomas.

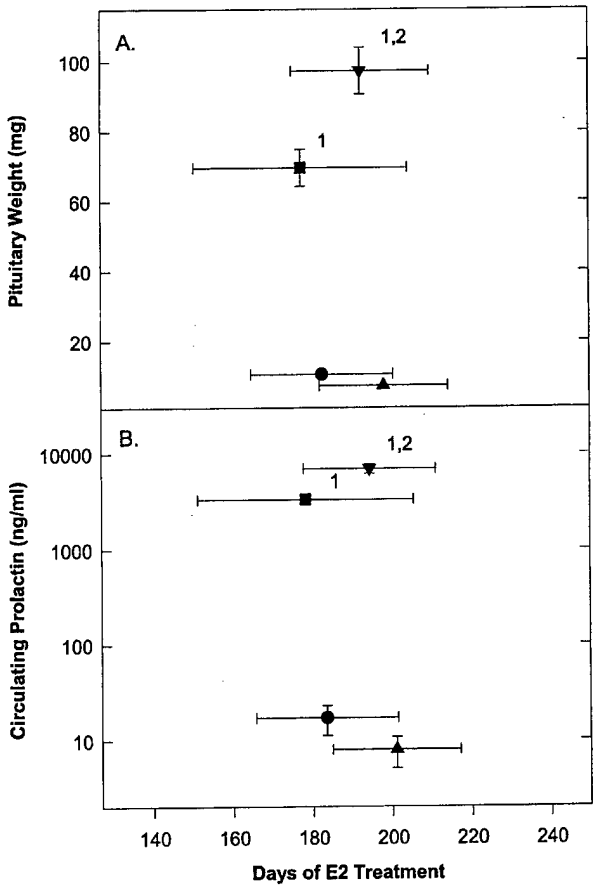


**Fig. 7.** Dietary energy restriction does not inhibit E2-induced pituitary tumorigenesis and associated hyperprolactinemia in ACI rats. Animals were treated as described in Figure 1 and the Materials and methods. (**A**) Each data point represents mean pituitary wet weight (± SEM, $n = 21$). Pituitary weight is a surrogate indicator of absolute lactotroph number. (**B**) Each data point represents the mean serum PRL level (± SEM, $n = 21$). The horizontal axis (A and B) represents the average duration of E2 treatment (± SD). Symbols: filled circle, control diet, untreated; filled square, control diet, E2 treated; filled triangle, energy restricted diet, untreated; filled inverted triangle, energy restricted diet, E2 treated. Numerals: 1, indicates a statistically significant difference ($P < 0.05$) between untreated and E2-treated animals fed the same diet; 2, indicates a statistically significant difference ($P < 0.05$) between similarly treated animals fed the different diets.

ACI rats fed the control diet (Figure 6A), whereas the number of cells staining positive for PR appeared to be reduced in the mammary glands of untreated animals fed the energy-restricted diet (Figure 6B). PR expression was also exhibited by a subset of cells in the hyperplastic mammary tissues of E2-treated rats fed either the control (Figure 6C) or energy-restricted diet (Figure 6D). Virtually all of the epithelial cells within the focal regions of atypical hyperplasia induced by E2 exhibited strong immunoreactivity to PR, regardless of whether these lesions exhibited secreta (Figure 6E and G), and dietary energy restriction did not affect PR expression in these atypical lesions (Figure 6F and H). Similarly, dietary energy restriction did not affect PR expression in E2-induced mammary carcinomas, in which nearly all of the cells exhibited strong immunoreactivity to PR (Figure 6I and J). The data strongly suggest that the inhibitory actions of dietary energy restriction on E2-induced mammary tumorigenesis are independent of PR expression.

*Dietary energy restriction does not inhibit estrogen-induced pituitary tumorigenesis and associated hyperprolactinemia*

In female ACI rats fed the control diet, the average pituitary weight was increased 6.7-fold, from 10.4 ± 0.2 mg (mean ± SEM) in untreated rats to 69.6 ± 0.5 mg in rats treated with E2 for 177 ± 26 (SD) days (Figure 7A). Pituitary weights of rats fed the energy-restricted diet and treated with E2 for 193 ± 19 days averaged 97.2 ± 6.6 mg, an increase of 13.5-fold over the 7.2 ± 0.1 mg observed in age-matched untreated rats fed the restricted diet. Treatment with E2 increased circulating PRL 190-fold, from 17 ± 6 ng/ml in untreated rats, to 3224 ± 70 ng/ml in female ACI rats fed the control

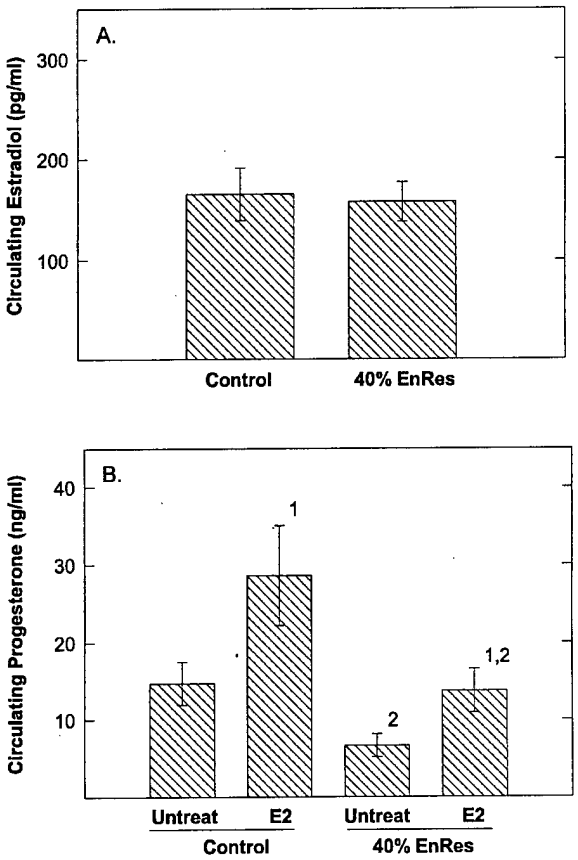


**Fig. 8.** Effects of treatments on circulating E2 and progesterone. Illustrated data are from rats treated with E2 for 84 days. (**A**) Each data bar represents the mean (± SEM, $n$ = 7–8) level of circulating E2 observed in E2-treated ACI rats fed either the control or the energy-restricted diet. E2 levels in untreated rats were not measured. (**B**) Each data bar represents the mean (± SEM, $n$ = 8) level of circulating progesterone levels. Numerals: 1, indicates a statistically significant difference ($P < 0.05$) between untreated and E2-treated animals fed the same diet; 2, indicates a statistically significant difference ($P < 0.05$) between similarly treated animals fed the different diets.

diet (Figure 7B). In animals fed the energy-restricted diet, circulating PRL levels increased 865-fold, from 8 ± 3 ng/ml in untreated rats, to 6921 ± 732 ng/ml in E2-treated rats. The data clearly indicate that dietary energy restriction does not inhibit the ability of administered E2 to induce development of PRL-producing pituitary tumors and associated hyperprolactinemia in ovary-intact ACI rats. In addition, these data exclude inhibition of pituitary PRL output as a potential mechanism through which dietary energy restriction inhibits mammary tumorigenesis.

*Analysis of circulating estradiol and progesterone*

When examined following 84 days of treatment, circulating E2 levels did not differ significantly between treated rats fed the control or the energy-restricted diet (Figure 8A). Circulating E2 levels in animals killed following varying lengths of E2 treatment ranging from 103 to 216 days in rats fed the control diet and from 158 to 216 days in rats fed the energy-restricted diet averaged 120 ± 13 and 238 ± 30 pg/ml, respectively. The observed levels of circulating E2 approximate those observed in the rat during pregnancy (36). Circulating E2 levels in untreated rats were not measured. During the rat estrous cycle, circulating E2 levels oscillate between ~20 and 75 pg/ml.

Treatment with E2 for 84 days resulted in an ~2-fold increase in the level of circulating progesterone, in ACI rats fed either the control or energy-restricted diet (Figure 8B). Interestingly, dietary energy restriction reduced circulating progesterone by ~50% in both untreated and E2-treated ACI rats, when examined at the 84 day time point. Circulating progesterone was also assayed in animals from the tumor study killed following varying lengths of E2 treatment. Progesterone levels in untreated and E2-treated rats fed the control diet averaged 13 ± 2 and 21 ± 3 ng/ml, respectively, whereas levels in untreated and E2-treated rats fed the energy-restricted diets averaged 9 ± 1 and 11 ± 2 ng/ml, respectively. Each of these observed levels of circulating progesterone is within the physiologic range. The data indicate that dietary energy restriction reduces circulating progesterone in both untreated and E2-treated ACI rats.

## Discussion

Data presented herein indicate that a 40% restriction of dietary energy consumption markedly inhibits mammary tumorigenesis in female ACI rats treated continuously with E2. Although dietary energy restriction has been demonstrated to inhibit mammary tumorigenesis in several other rodent models (4–8), this is the first demonstration that dietary energy restriction inhibits tumorigenesis in an animal model in which mammary cancers are induced solely by the administration of an estrogen, either naturally occurring or synthetic, or another hormonal agent. This study provides novel insights into the interactions between dietary and endocrine factors in the etiology of mammary cancer in a unique and physiologically relevant animal model.

In the present study, focal regions of atypical hyperplasia, which may be precursors to carcinomas, were common in the mammary glands of E2-treated ACI rats, regardless of whether the animals consumed the control or the energy-restricted diet. Therefore, we conclude that dietary energy restriction exerts its marked inhibitory effect on E2-induced mammary tumorigenesis at a stage subsequent to the development of atypical mammary epithelial hyperplasia. Zhu *et al.* (37) demonstrated that dietary energy restriction exerted a greater inhibitory effect on the number of MNU-induced mammary cancers in female Sprague–Dawley rats than on the frequencies of intraductal proliferations and ductal carcinoma *in situ*, both of which are believed to be precursor lesions in the MNU model. Therefore, energy restriction appears to attenuate or retard progression of pre-neoplastic lesions to carcinomas in both the E2-induced and MNU-induced models.

It has often been hypothesized that dietary energy restriction exerts marked inhibitory effects on tumorigenesis in different cancer models by inhibiting cell proliferation (7,9,11). Lok *et al.* (10) demonstrated a correlation between the inhibitory effects of differing degrees of dietary energy restriction (10–40%) on mammary tumorigenesis and mammary cell proliferation in female Swiss Webster mice. Sinha *et al.* (12) demonstrated that the marked inhibitory effect of a 50% restriction of food consumption on DMBA-induced mammary tumorigenesis in female Sprague–Dawley rats was associated with a significant reduction in the [$^3$H]thymidine labeling index in the mammary gland. Thompson *et al.* (8,38,39) demonstrated that dietary energy restriction reduced cell proliferation and

increased apoptosis in putative precursor lesions induced by MNU in the mammary glands of female Sprague–Dawley rats, and correlated these effects of energy restriction with reduced expression in the mammary epithelium of cyclin D1, an activator of G1 to S cell-cycle progression, and increased expression of $p27^{Kip1}$, an inhibitor of specific cyclin-dependent kinases. In the present study, a 40% restriction of dietary energy consumption significantly inhibited E2-induced mammary epithelial cell proliferation in the ACI rat, even in the presence of continuously elevated E2 and PRL. However, energy restriction did not block development of E2-induced lobuloalveolar hyperplasia or focal regions of atypical epithelial hyperplasia. Therefore, we consider it unlikely that this partial inhibition of E2-stimulated mammary cell proliferation directly contributed to the marked inhibitory effect of energy restriction on E2-induced mammary tumorigenesis observed in the present study. It is interesting to note that, in contrast to the observed inhibitory effect of dietary energy restriction on mammary cell proliferation in the E2-treated ACI rats, no inhibition of cell proliferation was observed within the E2-induced mammary tumors. The data differ from those of Thompson *et al.* (8,38) who observed a significant inhibitory effect of dietary energy restriction on cell proliferation within mammary adenocarcinomas induced by MNU in female Sprague–Dawley rats. These differing observations may result from genetic differences in the E2-induced and MNU-induced models or from endocrine related differences in the tumors and/or animals.

It has been suggested that one mechanism through which energy restriction may inhibit mammary tumorigenesis is by reducing output of ovarian estrogens and/or pituitary PRL (16–18). Sylvester *et al.* (40) demonstrated that administration of estradiol benzoate and/or haloperidol, a dopamine antagonist that stimulates PRL secretion, at least partially abrogates the inhibitory effect of a 50% restriction of food consumption on DMBA-induced mammary tumorigenesis in female Sprague–Dawley rats. Similarly, elevation of circulating PRL, produced by pituitary isografts, eliminates the inhibitory effect of a 30% restriction of energy consumption on mammary tumorigenesis in female C3H mice (15). A novel observation from the present study is that dietary energy restriction is capable of dramatically inhibiting mammary tumorigenesis in the ACI rat even when circulating E2 and PRL are maintained at high levels as a consequence of administration of exogenous E2.

We have demonstrated that ovariectomy markedly inhibits development of E2-induced mammary cancers in ACI rats and have interpreted this observation to suggest that rapid development of mammary cancers in E2-treated ACI rats requires the actions of one or more ovarian factors, in addition to E2 (25). Progesterone is one ovarian hormone that may be required for the rapid development of E2-induced mammary cancers in this animal model. Epidemiologic studies suggest a role for progesterone in the etiology of breast cancer in humans (20,41–44). As discussed above, both the focal regions of atypical epithelial hyperplasia and mammary cancers induced in ACI rats by E2 exhibit increased expression of PR, relative to the surrounding epithelium (26). In the present study, PR expression was not detectably affected by dietary energy restriction. However, energy restriction did significantly reduce the level of circulating progesterone in the E2-treated rats. At this time it is not known to what extent, if any, the observed reduction in circulating progesterone contributed to the inhibition of E2-induced mammary tumorigenesis by dietary energy restriction.

In summary, we have demonstrated that dietary energy restriction significantly inhibits E2-induced mammary tumorigenesis in the ACI rat as evidenced by reduced mammary cancer incidence and burden and increased latency to the appearance of the first palpable mammary cancer. This inhibition was tissue specific, associated with reductions in circulating progesterone and may result from an attenuation or retardation of the progression of atypical hyperplasia to carcinoma. Because of the well-documented role of E2 in the etiology of human breast cancers, we believe that this animal model provides a valuable tool for the study of interactions between dietary, hormonal and genetic factors in mammary tumorigenesis.

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# Diet-Gene Interactions in Estrogen-Induced Mammary Carcinogenesis in the ACI Rat[1,2]

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ABSTRACT It is well accepted that hormonal, dietary and genetic factors each influence breast cancer risk. However, the underlying mechanisms and the extent to which these factors interact are largely unknown. We have demonstrated that the female ACI rat exhibits a unique genetically conferred propensity to develop mammary cancers when treated with physiological levels of 17β-estradiol (E2). More recently, we have mapped to rat chromosome 5 a strong genetic modifier of susceptibility to E2-induced mammary cancers, termed estrogen-induced mammary cancer 1 (*Emca*1), and have identified potential *Emca*1 candidate genes. Because estrogens have been inextricably linked to the genesis of breast cancer in humans, the ACI rat model has the potential to reveal novel physiologically relevant insights into how the contributory actions of E2 are modified by specific dietary factors. In the present study, we have examined the ability of a 40% restriction of dietary energy consumption to inhibit E2-induced mammary carcinogenesis. The hypothesis tested was that energy restriction will inhibit mammary carcinogenesis even when circulating E2 remains elevated through administration of exogenous hormone. The data presented herein strongly suggest that energy restriction inhibits E2-induced mammary carcinogenesis in the ACI rat at least partly by retarding progression of atypical hyperplastic foci to carcinoma. J. Nutr. 131: 3087S–3091S, 2001.

KEY WORDS: • *ACI rat* • *estrogen* • *energy restriction* • *mammary cancer* • *pituitary*

Numerous studies implicate ovarian hormones as critical factors in the etiology of breast cancer (1–3). For example, oophorectomy before menopause was shown to reduce significantly the risk of breast cancer. Moreover, prophylactic treatment with the antiestrogen tamoxifen was shown in a recent clinical trial to reduce by approximately 50% the incidence of breast cancer in a population of women at high risk for this disease (4). Estrogens have been hypothesized to contribute to breast cancer etiology by increasing the rate of mammary cell proliferation, thereby promoting the accumulation of somatic mutations (5). The exact mechanism through which estrogens contribute to the development of breast cancer is not known.

Diet is another important determinant of breast cancer risk in human populations. Numerous prospective and case-control studies associate height, body mass index or both with breast cancer risk and provide indirect evidence that energy consumption and balance influence breast cancer development (6,7). Supporting these epidemiological data are numerous studies demonstrating that dietary energy restriction without malnutrition markedly inhibits mammary carcinogenesis in both rat and mouse models (8–12). Mechanisms postulated to explain how dietary energy restriction may inhibit mammary carcinogenesis include inhibition of mammary epithelial cell proliferation and reduction of circulating estrogen and prolactin, two hormones known to regulate mammary gland growth, differentiation and function (12–18).

The female ACI[4] rat provides a physiologically relevant and genetically defined animal model for studying diet-hormone interactions in mammary cancer development. Data from our laboratory demonstrate that continuous treatment with the naturally occurring estrogen, 17β-estradiol (E2), rapidly induces mammary cancers in ovary-intact ACI rats whereas mammary cancers rarely develop in the absence of exogenous estrogen in this strain (19; D.M.E. Harvell, T. E. Strecker, B. Xie, K. L. Pennington, R. D. McComb and J. D. Shull, University of Nebraska Medical Center, unpublished

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[4] Abbreviations used: ACI, AxC-Irish; E2, 17β-estradiol.

observations, 2001). Chronic E2 administration to the ACI rat results in the induction of lobuloalveolar hyperplasia, subsequent appearance of focal regions of atypical epithelial hyperplasia and ultimately, development of multiple independently arising mammary cancers (19,20). The mammary cancers induced by E2 are estrogen dependent and exhibit genomic instability, features commonly associated with human breast cancer (20,21). In addition, ovariectomy markedly inhibits development of mammary cancers in ACI rats treated with E2. More recently, we demonstrated that a majority of the epithelial cells within both the focal regions of atypical hyperplasia and the mammary carcinomas exhibit dramatic down-regulation of expression of Cdkn2a (22) and increased expression of progesterone receptor relative to the surrounding epithelium (20). These data suggest that the atypical hyperplasia may represent a precursor lesion to carcinoma in this rat model of E2-induced mammary cancer.

In contrast to the ACI rat, the genetically related Copenhagen rat is highly resistant to E2-induced mammary cancer development (23). Interestingly, the mammary epithelium of the ACI rat exhibits a more robust proliferative response to E2 than does that of the Copenhagen rat strain, suggesting a possible mechanism for the observed differences in the susceptibilities of these two rat strains to E2-induced mammary cancers (20). Ongoing genetic studies in our laboratory indicate that susceptibility to E2-induced mammary cancers is inherited as an incompletely dominant trait in progeny resulting from ACI x Copenhagen intercrosses (J. D. Shull, K. L. Pennington, T. M. Reindl, M. C. Snyder, T. E. Strecker, T. J. Spady, M. Tochacek and R. D. McComb, University of Nebraska Medical Center, unpublished observations, 2001). From these intercrosses we have mapped to rat chromosome 5 a locus, *Emca*1, that modifies susceptibility to E2-induced mammary cancers (M. Tochacek, T. M. Reindl, E. A. VanderWoude, C. R. Murrin and J. D. Shull, University of Nebraska Medical Center, unpublished observations, 2001). Residing within this locus is *Cdkn2a*, which has been implicated in the etiology of human breast cancer. We are presently investigating *Cdkn2a* as a candidate for the *Emca*1 modifier of susceptibility to E2-induced mammary cancers. The purpose of the present study was to examine the effects of dietary energy restriction on mammary carcinogenesis in this novel and physiologically relevant animal model. Data summarized herein demonstrate the ability of energy restriction to inhibit mammary carcinogenesis in the ACI rat, even when circulating E2 is maintained at a high level as a consequence of exogenous E2 administration.

## METHODS

The source of experimental animals, reagents and supplies as well as the methods relating to care, feeding and hormonal treatment of animals have been described previously (19,24–28). Ovary-intact ACI rats were obtained at age 42 d and individually housed. One week later, the animals were randomly assigned to experimental groups fed either a control or energy-restricted diet, defined as 40% reduction in total consumption of energy derived from fat and carbohydrate without reduction in consumption of essential micro- and macronutrients. At age 59 d, treatment with the naturally occurring estrogen, E2, was administered via silastic tubing implants. Thereafter, the rats were examined twice weekly for the presence of palpable mammary tumors and were killed when the largest mammary tumor reached 1.5–2:0 cm in diameter or if signs of morbidity were observed. At necropsy, the location and size of each tumor were recorded. The diameter for each tumor was determined in two perpendicular dimensions, and the tumor volume was calculated using the formula $V = 4/3\pi^3$, where r is half the average diameter. Mammary cell proliferation was assayed by BrdU immunohistochemistry as described previously (20). The levels of E2 (19), prolactin (20) and progesterone (29) in serum from trunk blood were measured by radioimmunoassay as described previously. Statistical significance was assessed using Student's *t* test and two-way ANOVA with Newman-Keul's post hoc test. Mammary tumor latency and incidence were analyzed by the log rank test for comparison of cumulative incidence curves and the Fischer's exact test, respectively. Values of $P < 0.05$ were considered statistically significant.

## RESULTS

***Dietary energy restriction inhibits estrogen-induced mammary carcinogenesis.*** Administration of E2 rapidly induced mammary cancers in ACI rats fed the control diet. In this population the first palpable mammary tumor was detected 69 d after the initiation of E2 treatment, and 100% of the animals exhibited one or more palpable mammary tumors within 216 d of E2 administration (**Table 1**). In contrast,

**TABLE 1**

*The effect of dietary energy restriction on E2-induced mammary carcinogenesis in the ACI rat*[1]

| | Control group | Energy-restricted group |
|---|---|---|
| Tumor incidence | | |
| At risk | 100% (21/21) | 59% (10/17)[2]* |
| Total | 100% (21/21) | 48% (10/21)* |
| Appearance of first tumor (days E2) | 69 | 104 |
| Latency (days E2) for tumor-bearing population | | |
| Median | 118 | 150* |
| Mean[3] | 126 ± 29 ($n$ = 21) | 154 ± 35 ($n$ = 10) |
| Average tumor, *n/rat* | 6.9 | 0.9* |
| Total tumor yield | 145 | 18* |
| Mean[4] tumor volume, *mm*[3] | 5,584 ± 772 | 458 ± 227* |
| Mean[3] length E2 treatment, *d* | 177 ± 26 (103–216)[5] | 193 ± 19 (158–216)[5] |

1 Where not indicated, values were assessed among animals in the total population.
2 Four animals exhibited morbidity, apparently because of pituitary tumors. These animals were killed 176, 188, 195 and 210 d after the initiation of E2 treatment and were tumor free at necropsy.
3 Mean ± standard deviation (SD).
4 Mean ± standard error of the mean (SEM).
5 Range given in parentheses.
* Significantly different from E2 treated ACI rats fed the control diet, $P < 0.01$.

among animals fed the energy-restricted diet and treated with E2 the first palpable mammary tumor was observed following 104 d of treatment, and 59% (10/17) of the population at risk had tumors by 207 d. Differences in both median latency and final mammary tumor incidence between the groups of E2 treated animals fed the control diet versus the energy-restricted diet ($P < 0.01$) were statistically significant. Differences in tumor number and volume observed in the E2-treated animals fed the control or energy-restricted diets were also statistically significant ($P < 0.01$). Five of 21 rats in the energy restricted, E2-treated group exhibited morbidity, apparently because of an E2-induced pituitary tumor, and were killed after 176, 188 (2 rats), 195 and 201 d of E2 treatment. Four of these animals were free of palpable mammary tumors at the time of death. Circulating E2 levels were equivalent in the E2-treated animals fed either diet. These data indicate that dietary energy restriction can reduce mammary tumor incidence, multiplicity and tumor size as well as increase the latency to the appearance of the first palpable mammary tumor in female ACI rats after continuous E2 treatment. Untreated ovary-intact ACI rats fed either the control or energy-restricted diet did not develop mammary tumors over the course of this experiment.

***Dietary energy restriction inhibits mammary cell proliferation in normal but not in neoplastic mammary tissue.*** To address the mechanism through which dietary energy restriction may inhibit E2-induced mammary carcinogenesis in the female ACI rat, we examined the effect of dietary energy restriction on mammary cell proliferation. Cell proliferation within the mammary epithelium was examined after 84 d of E2 treatment, a time preceding the appearance of most induced mammary tumors, and after 180–210 d of E2 treatment, a range of times when animals were being killed because of the presence of mammary tumors. In ACI rats fed the control diet, a marked stimulatory effect of E2 on mammary epithelial cell proliferation was evident at both the early and later times; the fraction of cells staining positive for BrdU was increased from approximately 0.5% in untreated rats to 3.5–4.0% in E2-treated rats. In the rats fed the energy-restricted diet, the ability of E2 to induce mammary cell proliferation was partially but significantly ($P < 0.01$) attenuated; the fraction of cells staining positive for BrdU was approximately 2% at both times.

We also examined the effects of dietary energy restriction on cell proliferation in E2-induced mammary tumors of the ACI rat. Approximately 7% of cells in E2-induced mammary tumors incorporated BrdU regardless of whether the rats consumed the control or the energy-restricted diet. This level of cell proliferation was significantly greater than in the surrounding hyperplastic mammary epithelium. Therefore, the inhibitory effect of dietary energy restriction on E2-induced mammary cell proliferation is restricted to normal mammary epithelium.

***Dietary energy restriction appears to retard progression of atypical hyperplasia to carcinoma.*** We previously demonstrated the presence of focal regions of atypical epithelial hyperplasia in the mammary tissue of ACI rats treated with E2 for as few as 12 wk (20). These atypical hyperplastic foci were characterized by an expanded acinus composed of cells exhibiting slightly enlarged nuclei and dense cytoplasmic staining. Interestingly, the focal regions of both the atypical epithelial hyperplasia and the mammary cancers induced in ACI rats by E2 exhibit increased expression of progesterone receptor relative to the surrounding epithelium, suggesting a link between these lesions (20). It was, therefore, of interest to determine whether consumption of an energy-restricted diet would inhibit the development of atypical hyperplastic foci or modulate progesterone receptor expression in ACI rats after E2 treatment. Focal regions of atypical epithelial hyperplasia were observed in the mammary glands of female ACI rats fed either the control or energy-restricted diet and treated with E2 for 12 wk. The number of these lesions increased as the treatment was extended beyond this time, and lesions were at least as common in the mammary glands of treated rats fed the energy-restricted diet as in treated rats fed the control diet. Histological examination revealed that the majority of the tumors that developed in the E2-treated animals were carcinomas of the comedo type regardless of whether the animals were fed the control or the energy-restricted diet. Carcinomas exhibiting invasive features were also observed in E2-treated animals regardless of the diet fed. These data suggest that dietary energy restriction exerts its marked inhibitory effect on E2-induced mammary carcinogenesis at a stage subsequent to the development of atypical mammary epithelial hyperplasia (Fig. 1).

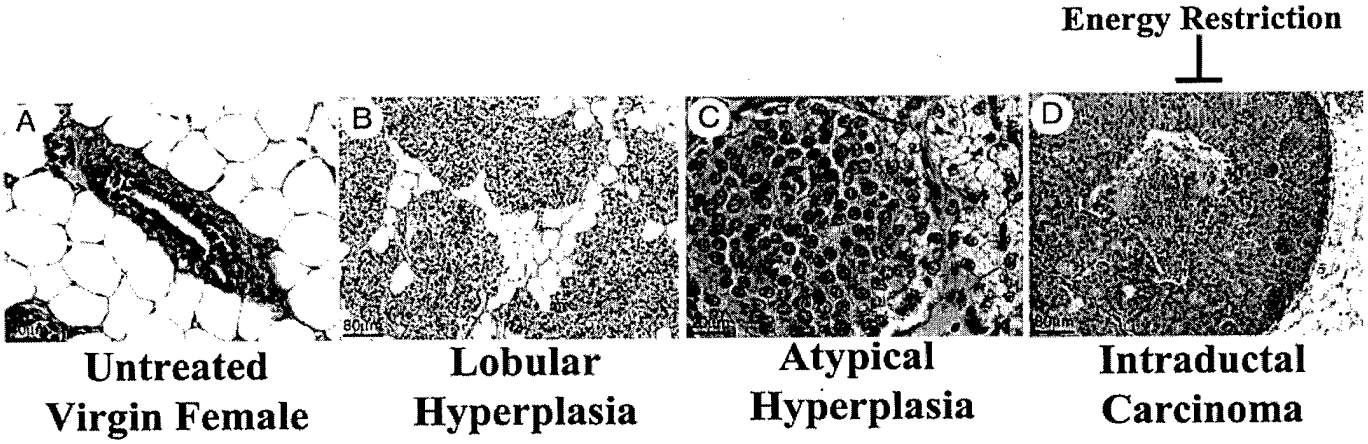


**FIGURE 1** Model of the inhibitory actions of dietary energy restriction on E2-induced mammary carcinogenesis. *A–D*: the progression of mammary carcinogenesis in ACI rats fed a control diet and treated with estrogen. Consumption of an energy-restricted diet appears to exert its inhibitory effects on E2-induced mammary carcinogenesis at a stage subsequent to the development of atypical mammary epithelial hyperplasia and may act by inhibiting the progression of this lesion to carcinoma.

Progesterone receptor expression was up-regulated within the focal regions of atypical hyperplasia and the carcinomas induced by E2 in animals fed either the control or the energy-restricted diet. From these data we conclude that inhibitory actions of dietary energy restriction on E2-induced mammary carcinogenesis are independent of progesterone receptor expression. Circulating progesterone, however, was reduced by dietary energy restriction, suggesting a possible mechanism for inhibition of carcinogenesis. It has also been suggested that energy restriction may inhibit mammary carcinogenesis by reducing output of pituitary prolactin, so circulating prolactin was also measured. Dietary energy restriction did not inhibit E2-induced pituitary tumorigenesis and associated hyperprolactinemia. Together, these data indicate that the inhibitory actions of energy restriction on E2-induced mammary carcinogenesis are independent of circulating levels of prolactin but may result in part from a reduction in circulating progesterone.

## DISCUSSION

The data described herein demonstrate that a 40% restriction of dietary energy consumption can inhibit mammary carcinogenesis in ovary-intact ACI rats treated continuously with E2. Although dietary energy restriction has been demonstrated to inhibit mammary carcinogenesis in several rat and mouse models (8–12), our findings are novel in that they demonstrate the ability of energy restriction to inhibit mammary carcinogenesis in an animal model where mammary cancer is induced solely through the actions of a naturally occurring hormone.

We do not know the mechanism through which dietary energy restriction inhibits E2-induced mammary cancer development. The inhibitory actions of energy restriction were associated with a reduction in E2-stimulated mammary cell proliferation. However, this inhibition was insufficient to block induction of lobuloalveolar hyperplasia or focal regions of atypical hyperplasia. These data suggest that dietary energy restriction inhibits E2-induced mammary cancers by attenuating the progression of atypical hyperplasia to carcinoma (Harvell et al. unpublished observations, 2001). This inhibition did not appear to result from a reduction in progesterone receptor expression, either in the atypical hyperplasia or carcinomas, but may result in part from reductions in circulating progesterone levels. Dietary energy restriction did not inhibit the ability of administered E2 to induce prolactin-producing pituitary tumors and associated hyperprolactinemia, indicating that the inhibitory effect of dietary energy restriction on mammary carcinogenesis is tissue specific and independent of circulating prolactin. This absence of an inhibitory effect of energy restriction on E2-induced pituitary tumorigenesis in ovary-intact ACI rats confirms and extends our previously published data demonstrating a lack of an effect of energy restriction on E2-induced pituitary tumorigenesis in ovariectomized ACI rats (27). In contrast, we (24–26,28) have demonstrated a marked inhibitory effect of dietary energy restriction on estrogen-induced pituitary tumorigenesis in the F344 rat strain. Together, these data indicate that the inhibitory actions of energy restriction on estrogen-induced pituitary tumorigenesis are strain specific and dependent on genetic background.

Because exogenous E2 is the sole inducing agent in this model, our study clearly demonstrates that the inhibitory effect of energy restriction on mammary carcinogenesis is downstream of potential effects of energy restriction on output of estrogens by the ovaries. However, inhibition of ovarian progesterone output by dietary energy restriction may contribute to the reduction in mammary cancers observed in the energy-restricted rats. Moreover, because the administered E2 maintains production of pituitary prolactin at a high level, we conclude that the ability of dietary energy restriction to inhibit mammary carcinogenesis is independent of any potential effect on pituitary prolactin output.

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# Phosphorylation Regulates the Nucleocytoplasmic Distribution of Kinase Suppressor of Ras*

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**KSR (kinase suppressor of Ras) has been proposed as a molecular scaffold regulating the Raf/MEK/ERK kinase cascade. KSR is phosphorylated on multiple phosphorylation sites by associated kinases. To identify potential mechanisms used by KSR to regulate ERK activation, green fluorescent protein was fused to intact and mutated KSR constructs lacking specific phosphorylation sites, and the subcellular distribution of each construct was observed in live cells. Mutation of a subset of KSR phosphorylation sites caused the redistribution of KSR to the nucleus. To determine whether intact KSR is normally imported to the nucleus, REF-52 fibroblasts expressing KSR were treated with 10 nM leptomycin B, which inhibits Crm1-dependent nuclear export. KSR accumulated in the nucleus within 2 h of treatment with leptomycin B, suggesting that KSR cycles continuously through the nucleus. Nuclear import of KSR was blocked by mutations that inhibit the interaction of KSR with MEK. Coexpression of fluorescent forms of KSR and MEK in cells revealed that each protein promoted the localization of the other in the cytoplasm. These data indicate that the subcellular distribution of KSR is dynamically regulated through phosphorylation and MEK interaction in a manner that may affect signaling through ERK.**

Ras proteins are small GTPases that mediate extracellular signals to control cell growth and differentiation (1–3). Activated Ras proteins are also the most common oncoproteins detected in human cancers, including cancers of the lung, pancreas, and colon. A major function of the Ras protein is transmission of mitogenic signals from the plasma membrane to the nucleus through the Raf/MEK[1]/ERK kinase cascade, also termed the MAPK pathway. Genetic screens for modifiers of Ras signaling in *Caenorhabditis elegans* and *Drosophila melanogaster* identified KSR as a positive regulator of Ras signaling, functioning between Ras and Raf or in a pathway parallel to Raf (4–6). Consistent with these epistasis experiments, structure/function analysis revealed that a cysteine-rich region (CA3) in KSR enhances the positive effects of Ras on Raf (7, 8). However, KSR may also regulate other points in the MAPK pathway. KSR binds Raf, MEK, and ERK (7, 9–12). Interaction of the KSR kinase domain with MEK is biologically important, as a mutation that prevents the interaction of KSR and MEK (12, 13) also disrupts KSR function in *C. elegans* (5). These data have led to the hypothesis that KSR functions as a scaffold to coordinate these components of the kinase cascade (7, 9–12, 14). Characteristic of a molecular scaffold (15), overexpression of ectopic KSR inhibits signaling through the MAPK cascade (7, 11, 14, 16).

Regulating the subcellular distribution of signaling components is one way a scaffold can control signaling through an intracellular pathway. Translocation of Raf, MEK, and ERK to different subcellular compartments is essential to their normal function. Raf interacts with Ras in a GTP-dependent manner and translocates to the plasma membrane (17–23). ERKs facilitate gene transcription by translocating to the nucleus, phosphorylating transcription factors, and stimulating their activity (24–27). Similar to ERKs, MEK translocates to the nucleus upon stimulation with mitogens or serum (28, 29). Evidence also suggests that MEK and ERK, rather than localizing exclusively to the cytoplasm in resting cells, are continuously cycling in and out of the nucleus (30, 31). Nuclear export of ERK involves a MEK-dependent active transport mechanism (32). The ability of KSR to interact with Raf, MEK, and ERK and observations that loss-of-function mutations in KSR specifically disrupt KSR/MEK interaction raise the possibility that KSR may affect signaling through the MAPK cascade by altering the subcellular distribution of one or more signaling components.

To better understand the mechanism by which KSR regulates the MAPK pathway, we used a GFP-KSR fusion protein to determine the subcellular distribution of KSR in live cells. We found that KSR was localized to the cytoplasm in quiescent cells, but, like MEK and ERK, continuously cycled through the nucleus. Mutations in KSR that altered its ability to bind MEK also inhibited nucleocytoplasmic transport of KSR. We also found that phosphorylation of KSR regulated its nucleocytoplasmic distribution, but did not impair its association with

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[1] The abbreviations used are: MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; KSR, kinase suppressor of Ras; GFP, green fluorescent protein; YFP, yellow fluorescent protein; CFP, cyan fluorescent protein; NES, nuclear export signal; NLS, nuclear localization signal; Raf-CAAX, c-Raf-1 kinase targeted to the plasma membrane by a carboxyl-terminal lipid modification signal from Ha-Ras; MBP, myelin basic protein.

MEK. This study suggests a link between the ability of KSR to translocate to the nucleus, to bind MEK, and to regulate ERK activation.

## MATERIALS AND METHODS

*Cell Culture*—Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and incubated at 37 °C. 293T and REF-52 cells were grown in a 5% $CO_2$ atmosphere.

*Construction of KSR Expression Plasmids*—The addition of the FLAG epitope tag to the COOH terminus of KSR and the construction of pCMV5KSR.R589M, pCMV5KSR.T411V, pCMV5KSR.S518A, pCMV5KSR.S392A, and pCMV5KSR.T274V were described previously (14, 33). To create pCMV5KSR.S518A/T411V, pCMV5KSR.S518A and pCMV5KSR.T411V were digested with *Bst*XI, and the appropriate fragments were ligated together. pCMV5KSR.T274V/T411V was created by digesting pCMV5KSR.T274V and pCMV5KSR.T411V with *Bam*HI and ligating together. 4mut is S518A/T274V/S392A/T411V. 5mut adds S297A; 6mut adds S190A; and 9mut adds S320A, S429A, S430A, S431A, S434A, and S435A. These constructs were created as described previously (33). pCMV5KSR.C809Y was created using the method described above and primer 5′-GGCGAGATCCTGTCTGCATACTGGGCTTTCGATCTGC-3′ (deletes the *Bgl*I site). Using pCMV5KSR.9mut, $Val^{274}$ was reverted to Thr using primer 5′-GCTGAAGCCACCAAGGACACCCCCACCGCC-3′ (deletes the created *Avr*II site) either singly or in combination with reversion of $Ala^{392}$ to Ser using primer 5′-CGGAGGACAGAGTCTGTCCCGTCAGATATC-3′ (destroys the created *Kpn*I site) to create 9mutA392S, 9mutV274T, and 9mutV274T/A392S. The remaining double mutants were created by PCR mutagenesis using one mutant as a template for mutation of the second site.

*Construction of GFP-KSR Fusion Proteins and Nuclear Targeted Proteins*—The GFP-KSR fusion constructs were created by digesting pEGFP-C1 (CLONTECH) and the construct in pCMV5 with *Eco*RI and *Kpn*I (wild-type KSR and R589M), *Xba*I (C809Y), *Bam*HI (9mut), or *Sal*I (T274V, S392A, S518A, T411V, S518A/S392A, T274V/S297A, T274V/S518A, S297A/S392A, S392A/T411V, 4mut, 5mut, 6mut, T274V/S392A, S518A/T411V, T274V/T411V, 9mutA392S, 9mutV274T, and 9mutV274T/A392S) and ligating the appropriate fragments together.

The sequence PKKKRKV was fused to the carboxyl terminus of KSR, KSR.R589M, and KSR.C809Y using primer 5′-GACTACAAGGACGACGATGACAAGCCTAAGAAGAAGCGGAAGGTCGACTAGGGTACCGG-3′ (34). The primer sequence contains a *Sal*I restriction site for screening.

*Construction of YFP-MEK Fusion Proteins*—The sequence of MEK1 was obtained by designing primers for PCR of the sequence from pcDNA1MEK1 (gift from Nick Grammatakakis), with enzyme sites *Hin*dIII (5′-end) and *Bam*HI (3′-end). MEK1 was then subcloned into pEYFP-C1 using *Hin*dIII/*Bam*HI. To construct YFP-MEK1.ΔNES, the reported NES (amino acids 32–44) (28) was deleted from YFP-MEK1 by PCR mutagenesis. The forward primer sequence (and its reverse complement) used was 5′-CAGCTCGGCCGAGACCAACCTGGAGCAGCAGCGGAAGCGGCTAGAGGCCTTTC-3′, and ALQKKLEELELDE (nucleotides 134–174) of MEK1 was deleted. The *Xho*I site was deleted, and the absence of this site was used to identify the appropriate construct.

The coding region for CFP was excised from the promoterless vector pECFP-1 and substituted for GFP in the pEGFP-C1 vector using the enzyme sites *Age*I and *Bsr*GI. CFP fusion proteins with KSR were constructed as described above to create GFP-KSR fusion proteins.

*ERK Assay*—293T cells were transfected with the indicated cDNAs using calcium phosphate (33). This procedure resulted in transfection efficiencies in 293T cells near 90%. Activation of the ERK MAPK was achieved by transfection of constitutively active Raf (Raf-*CAAX*). The cells were lysed, and clarified cell lysates were immunoprecipitated using anti-ERK1 and anti-ERK2 antibodies (Santa Cruz Biotechnology). The ERK assay was performed using MBP as a substrate (14). Reactions were stopped by the addition of sample buffer and heating to 110 °C for 3 min and then subjected to SDS-polyacrylamide gel electrophoresis on a 12% gel. Radiolabeled MBP was quantified using storage phosphor technology (Molecular Dynamics, Inc.).

*Western Blot Analysis*—Proteins were transferred to polyvinylidene difluoride membranes, and Western blots were developed using anti-FLAG antibody M2 (1:2000; Kodak Scientific Imaging Systems), anti-c-Raf antibody (1:1000; Transduction Laboratories), anti-GFP antibody (1:500; CLONTECH), anti-MEK antibody (1:1000; Transduction Laboratories), or phospho-specific anti-ERK antibody (1:1000; New England Biolabs Inc.). Blots were developed with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium using goat anti-mouse or goat anti-rabbit secondary antibody conjugated to alkaline phosphatase.

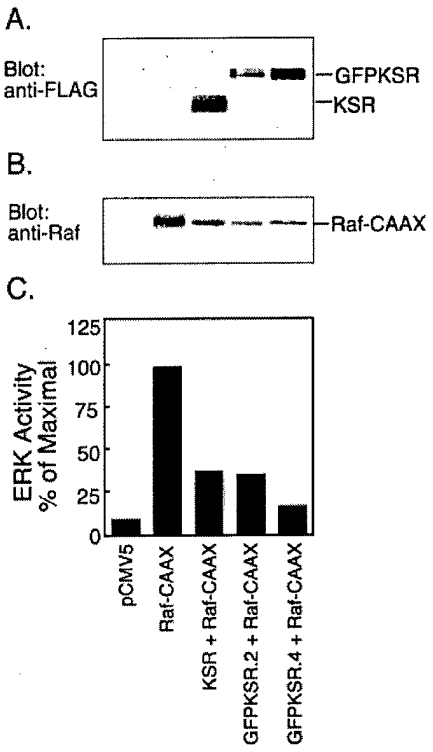


FIG. 1. **GFP-KSR inhibits ERK activity.** ERK kinase assays were performed with immunoprecipitates of 293T cells expressing activated Raf (Raf-*CAAX*) with or without pCMV5KSR or two different clones of GFP-KSR. *A*, Western blotting was performed with anti-FLAG antibody M2 to confirm the expression of KSR-FLAG. *B*, Western blotting was performed to confirm the expression of Raf-*CAAX*. *C*, 293T lysates expressing the indicated plasmids were immunoprecipitated with antibodies to ERK1 and ERK2, and the immunoprecipitates were incubated with [$^{32}$P]ATP and a generic substrate (MBP). Phosphorylation of MBP was measured by storage phosphor technology.

*Cell Microinjection*—REF-52 cells were either microinjected using an Eppendorf Transjector 5246 system or transfected using Superfect (QIAGEN Inc.) according to the manufacturers' instructions. Microinjected cells were serum-starved for 18 h before microinjection with 100 ng of plasmid/μl and examined by fluorescence microscopy 5 h after microinjection using the appropriate filters (Nikon, Inc.). Transfected cells were analyzed by fluorescence microscopy with the appropriate filter 18 h after transfection.

## RESULTS

*KSR Undergoes Nucleocytoplasmic Transport*—To gain insight into the molecular mechanism by which KSR regulates ERK activation, the subcellular location of KSR was determined. To detect KSR in live cells, GFP was fused to the amino terminus of KSR (GFP-KSR). We had previously observed the ability of ectopic KSR to inhibit ERK activation (14). To confirm that the fusion of GFP did not alter the ability of KSR to regulate ERK activity, GFP-KSR was cotransfected with Raf-*CAAX* (a constitutively active form of Raf) into 293T cells, and ERK activity was measured in an immune complex kinase assay. Ectopic expression of GFP-KSR inhibited ERK activity in a manner similar to KSR (Fig. 1), suggesting that the fusion of GFP does not affect the ability of KSR to regulate ERK activity. Fluorescence microscopy revealed that, at steady state, GFP-KSR microinjected into REF-52 cells was located in the cytoplasm in the majority of cells (Fig. 2*B*). pCMV5KSR was microinjected into REF-52 cells, and the cells were fixed and stained with anti-FLAG (M2) primary and anti-fluorescein isothiocyanate secondary antibodies. GFP-KSR had the same

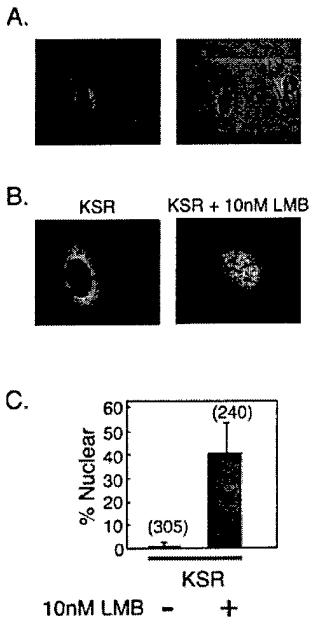


FIG. 2. **KSR cycles through the nucleus.** *A*, REF-52 cells were microinjected with a plasmid encoding FLAG-tagged KSR. KSR was detected within fixed cells with anti-FLAG antibody and a fluorescent secondary antibody (*left panel*). A phase-contrast photomicrograph of the injected cell (*left*) and an uninjected cell (*right*) are shown in the *right panel*. *B*, REF-52 cells were microinjected with a plasmid encoding GFP-KSR. Confocal microscopy was performed to determine KSR localization (*left panel*). REF-52 cells expressing GFP-KSR were treated with 10 nM leptomycin B (*LMB*) for 2 h, and confocal microscopy was performed to determine KSR localization (*right panel*). *C*, the cells described for *B* were scored, and the percentage of cells with nuclear fluorescence is shown. The number of cells scored under each condition is shown above each *bar*. Data are presented as the means ± S.D. of four independent experiments for untreated cells and five independent experiments for cells treated with leptomycin B.

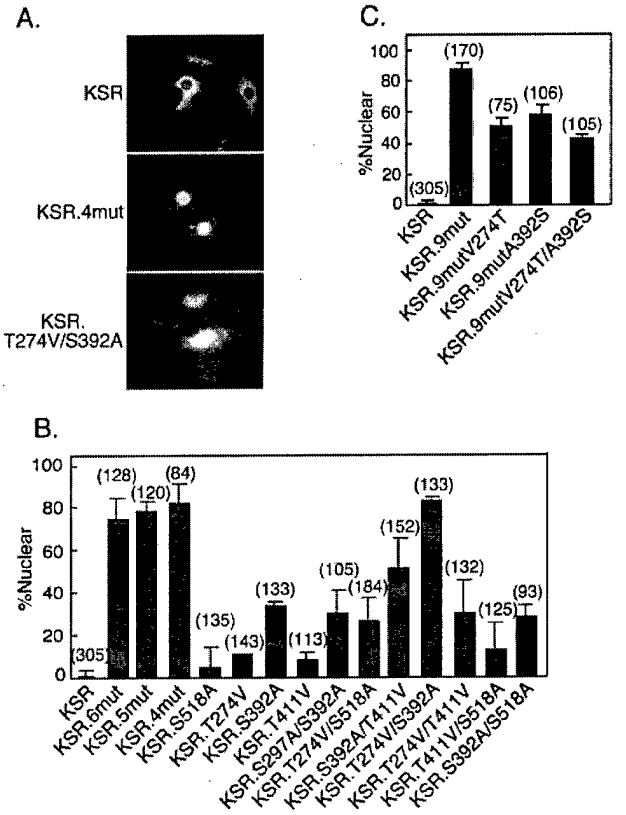


FIG. 3. **Phosphorylation regulates the nucleocytoplasmic distribution of KSR.** *A*, REF-52 cells were microinjected with expression plasmids encoding GFP-KSR (*upper panel*), GFP-KSR.4mut (S518A/S392A/T411V/T274V; *middle panel*), and GFP-KSR.T274V/S392A (*lower panel*). Fluorescence microscopy was used to detect each GFP-KSR protein. *B*, cells expressing KSR and KSR with the indicated mutated phosphorylation sites were scored, and the percentage of cells with nuclear fluorescence is shown. The number of cells scored for each mutant is indicated in *parentheses* above each *bar*. Results are presented as the means ± S.D. of three independent experiments for each construct. The S297A and S190A/S297A mutations were added to 4mut to construct 5mut and 6mut, respectively. *C*, the $Thr^{274}$ or $Ser^{392}$ phosphorylation site or both $Thr^{274}$ and $Ser^{392}$ were reintroduced to the backbone of a KSR construct containing nine mutated phosphorylation sites (KSR.9mut). KSR.9mut contains the mutated phosphorylation sites S429A, S430A, S431A, S320A, S434A, and S435A, in addition to the mutated sites contained in KSR.6mut as described above. Cells expressing KSR.9mut, KSR.9mutV274T, KSR.9mutA392S, or KSR.9mutV274T/A392S were scored, and the percentage of cells with nuclear fluorescence is shown. The total number of cells scored for each mutant is shown above each *bar*. Results are presented as the means ± S.D. of three independent experiments.

cytoplasmic distribution as KSR without GFP attached (Fig. 2*A*).

A small number of REF-52 cells expressing GFP-KSR had nuclear fluorescence. This observation led us to determine whether wild-type KSR undergoes nucleocytoplasmic shuttling. To test this possibility, we treated REF-52 cells expressing GFP-KSR with leptomycin B, an inhibitor of Crm1-mediated nuclear export (35). Upon treatment with 10 nM leptomycin B for 2 h, ~40% of the cells had nuclear fluorescence (Fig. 2, *B* and *C*); and importantly, few of the cells contained GFP-KSR exclusively in the cytoplasm. The localization of GFP alone did not change upon treatment with leptomycin B in control experiments (data not shown). This observation demonstrates that KSR cycles continuously in and out of the nucleus.

*Mutation of Phosphorylation Sites T274V and S392A Causes Nuclear Localization of KSR*—KSR is phosphorylated on multiple phosphorylation sites (16, 33). To determine whether phosphorylation of KSR regulates its subcellular localization, nine of the phosphorylation sites were mutated to non-phosphorylatable residues, and this mutant (GFP-KSR.9mut) was microinjected into REF-52 cells. Fluorescence microscopy revealed that KSR was located in the nucleus (data not shown). To ascertain which phosphorylation sites are important for nucleocytoplasmic transport, GFP fusion constructs of KSR mutants lacking six (6mut), five (5mut), and four (4mut) of the previously identified phosphorylation sites were created and expressed in REF-52 cells. The cells were then scored for nuclear fluorescence (Fig. 3*B*). Intact KSR was localized exclusively to the cytoplasm (Fig. 3*A*). However, 75–80% of the cells expressing 6mut, 5mut, or 4mut showed nuclear localization (Fig. 3*B*).

To determine which of the phosphorylation sites in GFP-KSR.4mut (T274V/S392A/T411V/S518A) are most important for nuclear localization, all possible single and double phosphorylation mutants were created and expressed in REF-52 cells. The most dramatic shift was seen in cells expressing T274V/S392A, with 80% of the cells showing nuclear fluorescence (Fig. 3, *A* and *B*). The individual mutations T274V and S392A had a modest ability to cause nuclear localization of KSR (Fig. 3*B*). However, a dramatic redistribution of KSR from the cytoplasm to the nucleus was seen upon combining these two mutations.

To determine whether $Thr^{274}$ and $Ser^{392}$ are the sole determinants of nuclear translocation, $Thr^{274}$ and $Ser^{392}$ were reintroduced both individually and in combination to the backbone of KSR.9mut and expressed in REF-52 cells. Reintroduction of the phosphorylation sites individually (KSR.9mutV274T and

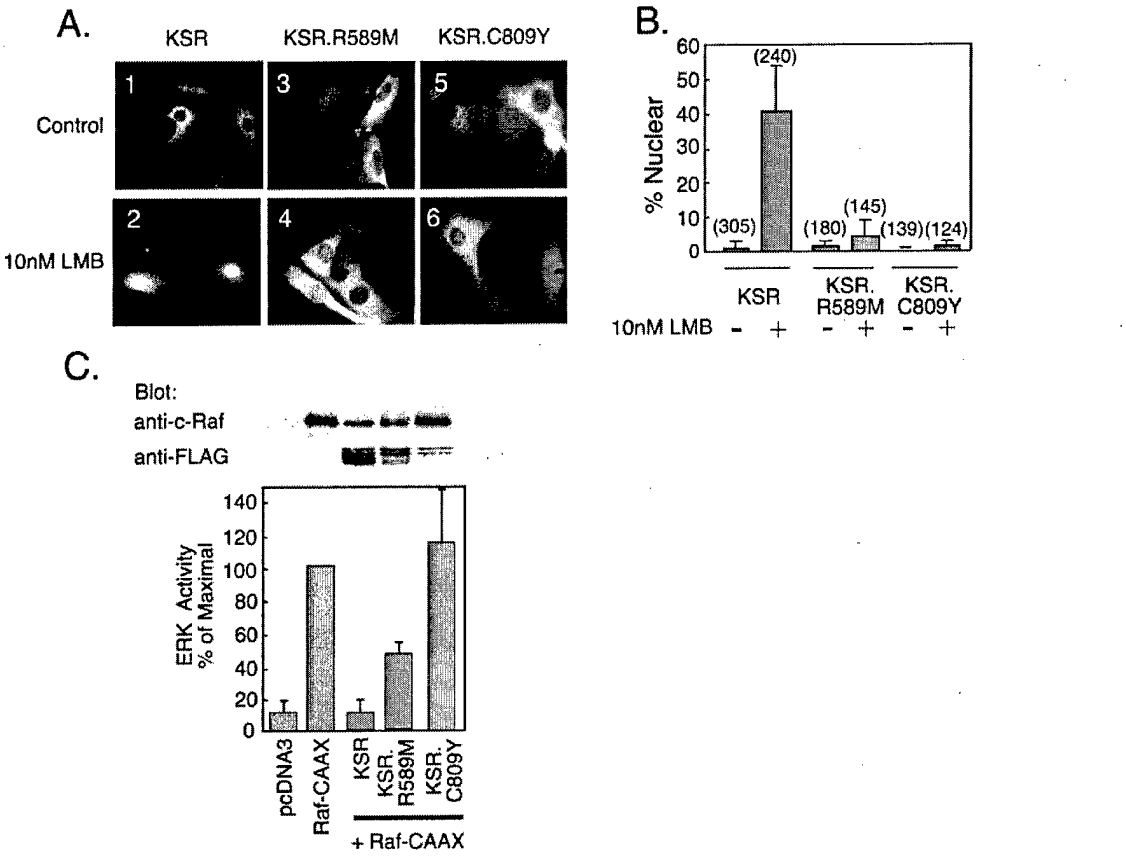


FIG. 4. **Mutations R589M and C809Y prevent nuclear import of KSR and reverse the inhibition of ERK by KSR.** *A*, REF-52 cells were microinjected with an expression plasmid encoding GFP-KSR (*panels 1* and *2*), GFP-KSR.R589M (*panels 3* and *4*), or GFP-KSR.C809Y (*panels 5* and *6*). The cells were left untreated (*panels 1*, *3*, and *5*) or treated with 10 nM leptomycin B (*LMB*) for 2 h (*panels 2*, *4*, and *6*). *B*, cells treated as described for *A* were scored, and the percentage of cells with nuclear fluorescence is shown. The number of cells scored for each condition is shown above each *bar*. Data are presented as the means ± S.D. of at least three independent experiments. *C*, 293T cells were transfected with control vector (pcDNA3) or with Raf-*CAAX* alone and in combination with KSR, KSR.R589M, and KSR.C809Y. Expression of transiently expressed proteins was confirmed by immunoprecipitation and Western blotting of cell lysates with antibodies to the FLAG epitope tag added to KSR or with anti-c-Raf antibodies. ERK activity was determined by phosphorylation of MBP added to immunoprecipitates of ERK1 and ERK2. MBP phosphorylation was quantified by storage phosphor technology. Results are presented as the means ± S.D. of three independent experiments.

KSR.9mutA392S) or together (KSR.9mutV274T/A392S) reduced the nuclear localization of KSR by 20–40%, but did not completely prevent its nuclear transport (Fig. 3*C*). These data suggest that $Thr^{274}$ and $Ser^{392}$ are potent regulators of KSR subcellular localization, but they are not the sole determinants of KSR nucleocytoplasmic transport.

*Mutations R589M and C809Y Abrogate Nucleocytoplasmic Shuttling*—The KSR kinase domain binds MEK (10–13). KSR.R589M has reduced ability to bind MEK1, and KSR.C809Y does not bind wild-type MEK1 (12, 13). KSR.R589M is a predicted kinase-dead mutation (7), and KSR.C809Y is analogous to a loss-of-function mutation of KSR identified in *C. elegans* (5). To determine whether these mutations in KSR affect nucleocytoplasmic transport, REF-52 cells expressing GFP-KSR.R589M and GFP-KSR.C809Y were treated with 10 nM leptomycin B for 2 h. In quiescent REF-52 cells, GFP-KSR.R589M and GFP-KSR.C809Y were located in the cytoplasm (Fig. 4*A*, *panels 1*, *3*, and *5*). In contrast to wild-type KSR, treatment with leptomycin B failed to significantly increase the nuclear accumulation of GFP-KSR.C809Y; however, there was a slight increase in nuclear accumulation in cells expressing GFP-KSR.R589M (Fig. 4, *A*, *panels 2*, *4*, and *6*; and *B*). These data indicate that mutations R589M and C809Y inhibit the nucleocytoplasmic shuttling of KSR.

To determine whether there is a correlation between regulation of ERK activity by KSR and the ability of KSR to shuttle through the nucleus, we assessed the ability of KSR.R589M and KSR.C809Y to regulate ERK activation. KSR, KSR.R589M, and KSR.C809Y were cotransfected with Raf-*CAAX* into 293T cells, and ERK activity was measured. Wild-type KSR inhibited 90% of ERK activation by Raf-*CAAX*; ectopic expression of KSR.R589M inhibited only 50% of ERK activation; and KSR.C809Y had no effect on ERK activation (Fig. 4*C*).

We further assessed the dependence of nuclear translocation of KSR on its interaction with MEK by creating a KSR construct containing the R589M, T274V, and S392A mutations. We observed that R589M was dominant with respect to the nuclear localizing effects of the phosphorylation site mutations (Fig. 5). These data suggest a relationship between the subcellular distribution of KSR, its ability to interact with MEK, and its ability to regulate ERK activity.

*Addition of the SV40 NLS Targets KSR to the Nucleus*—The sequence PKKKRKV from the SV40 large T antigen (34) was spliced by PCR to the carboxyl-terminal end of CFP-KSR (CFP-KSR.NLS), CFP-KSR.R589M (CFP-KSR.R589MNLS), and CFP-KSR.C809Y (CFP-KSR.C809YNLS) (Fig. 6*A*). These constructs were then expressed in REF-52 cells, and their subcellular localization was detected by fluorescence microscopy. All three NLS constructs were located exclusively in the nucleus (Fig. 6*B*).

To determine whether the nuclear localization of KSR alters

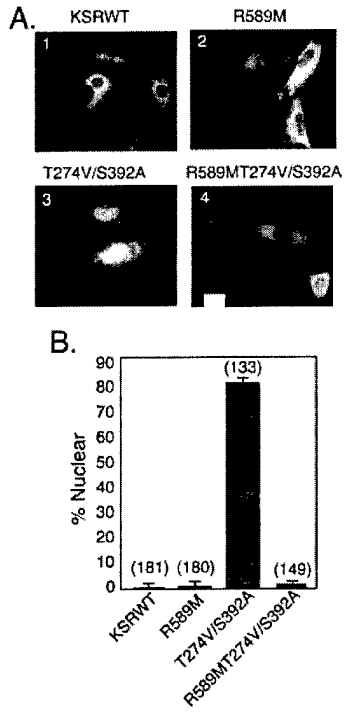


FIG. 5. **Mutation R589M prevents nuclear localization of KSR lacking phosphorylation sites Thr$^{274}$ and Ser$^{392}$.** *A*, GFP fusion constructs of wild-type KSR (*KSRWT*), KSR.R589M, KSR.T274V/S392A, and KSR.R589M/T274V/S392A were expressed in REF-52 cells, and their subcellular distribution was determined by fluorescence microscopy. *B*, cells expressing the KSR constructs shown in *A* were scored, and the percentage of cells with nuclear fluorescence is shown. The number of cells scored for each mutant is indicated in *parentheses* above each *bar*. Results are presented as the means ± S.D. of three independent experiments for each construct.

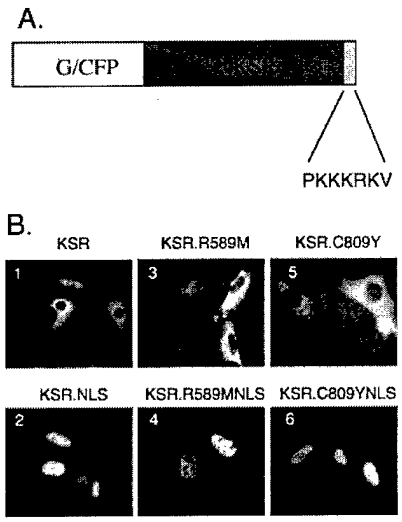


FIG. 6. **NLS from the SV40 large T antigen causes nuclear localization of KSR, KSR.R589M, and KSR.C809Y.** *A*, the NLS (PKKKRKV) from the SV40 large T antigen was spliced to the carboxyl terminus of the indicated fluorescent protein-KSR fusion constructs. *B*, CFP-KSR.NLS, CFP-KSR.R589MNLS, and CFP-KSR.C809YNLS were expressed in REF-52 cells (*panels 2*, *4*, and *6*), and their subcellular localization was compared with that of the constructs without the NLSs (*panels 1*, *3*, and *5*).

its ability to inhibit ERK activation, we performed ERK kinase assays on lysates from cells transfected with activated Raf-*CAAX* alone or with KSR constructs targeted to the nucleus. Mutation of phosphorylation sites on KSR that redistribute

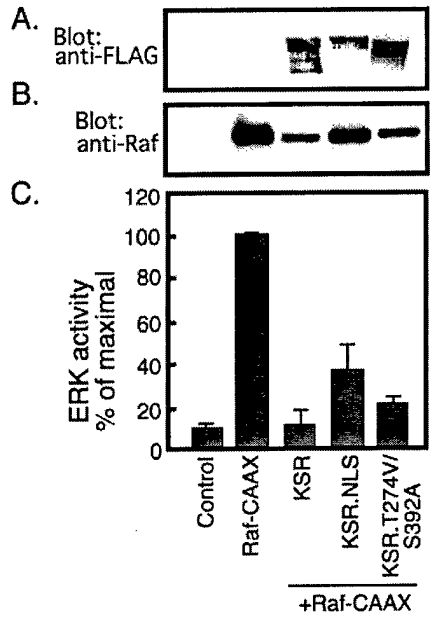


FIG. 7. **ERK activation in cells expressing KSR constructs targeted to the nucleus.** *A*, Western blotting was performed with anti-FLAG antibody M2 to confirm the expression of KSR-FLAG. *B*, Western blotting was performed to confirm the expression of Raf-*CAAX*. *C*, 293T lysates expressing the indicated plasmids were immunoprecipitated with antibodies to ERK1 and ERK2, and the immunoprecipitates were incubated with [$^{32}$P]ATP and a generic substrate (MBP). Phosphorylation of MBP was measured by storage phosphor technology.

KSR to the nucleus or the addition of an NLS to wild-type KSR had little if any effect on its ability to inhibit ERK activation (Fig. 7).

*MEK1 Re-localizes KSR.NLS and KSR.T274V/S392A to the Cytoplasm*—It has been reported that MEK accumulates in the nucleus upon treatment with leptomycin B (31), suggesting that MEK undergoes nucleocytoplasmic transport. Because KSR also undergoes nucleocytoplasmic transport and KSR and MEK interact, we sought to determine whether MEK plays a role in the nucleocytoplasmic transport of KSR. We analyzed the effect of MEK1 coexpression on the subcellular localization of KSR constructs targeted to specific subcellular compartments. To perform this analysis, YFP was fused to the amino terminus of MEK1, and CFP was added to the amino terminus of each KSR construct. This strategy enabled us to determine the subcellular location of MEK1 and KSR proteins simultaneously. When expressed together, YFP-MEK1 and CFP-KSR were located in the cytoplasm (data not shown). KSR.NLS constructs were coexpressed with YFP-MEK1 to evaluate the effect of MEK1 on KSR subcellular distribution. YFP-MEK1 remained in the cytoplasm when coexpressed with all KSR.NLS constructs and the KSR phosphorylation mutant CFP-KSR.T274V/S392A (Fig. 8, *A* and *C*). However, in the same cells, expression of YFP-MEK1 caused a marked redistribution of CFP-KSR.T274V/S392A from the nucleus to the cytoplasm (Fig. 8*C*). Furthermore, coexpression of YFP-MEK1 with CFP-KSR.NLS caused a redistribution of CFP-KSR.NLS from the nucleus to the cytoplasm. When coexpressed with YFP-MEK1, CFP-KSR.NLS was nuclear in only 25% of the cells compared with 98% of the cells when CFP-KSR.NLS was expressed alone (Fig. 8*D*). These data suggest that MEK could enhance nuclear export and/or inhibit nuclear import of KSR. However, additional data suggest that it is unlikely that interaction with MEK inhibits KSR nuclear import. First, CFP-KSR.NLS accumulated in the nucleus of cells cotransfected with YFP-MEK1 and treated with leptomycin B (data not

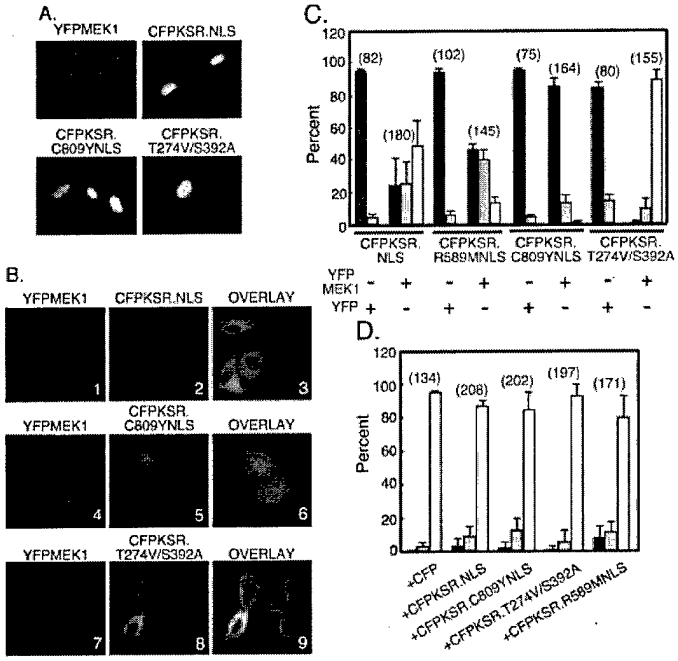


FIG. 8. **MEK1 re-localizes KSR.NLS and KSR.T274V/S392A from the nucleus to the cytoplasm.** *A*, shown are fluorescence photomicrographs of YFP-MEK1, CFP-KSR.NLS, CFP-KSR.C809YNLS, and CFP-KSR.T274V/S392A. *B*, CFP-KSR.NLS, CFP-KSR.C809YNLS, and CFP-KSR.T274V/S392A were expressed with YFP-MEK1 in REF-52 cells, and fluorescence microscopy was performed to detect the location of the YFP and CFP constructs simultaneously. The *red* pseudocolor (*panels 1*, *4*, and *7*) indicates the location of YFP-MEK1, and the *green* pseudocolor (*panels 2*, *5*, and *8*) reveals the location of the indicated CFP-KSR constructs. *Panels 3*, *6*, and *9* are the overlays of YFP and CFP fluorescence. *C*, the indicated CFP-KSR constructs were expressed with YFP alone or in combination with YFP-MEK1. The location of the indicated CFP-KSR constructs was detected by fluorescence microscopy, and cells were scored and grouped into one of three categories based on the following parameters: nuclear if the nucleus was brighter than the cytoplasm (*dark gray bars*), nuclear and cytoplasmic if the fluorescence was distributed evenly between the nucleus and cytoplasm (*light gray bars*), and cytoplasmic if the cytoplasm was brighter than the nucleus (*white bars*). The total number of cells scored for each construct is shown in *parentheses* above each *bar*. Results are presented as the means ± S.D. of at least three independent experiments. *D*, YFP-MEK1 was expressed with CFP or the indicated CFP-KSR constructs. The location of YFP-MEK1 was detected by fluorescence microscopy. Cells were scored and grouped into one of three categories described for *C*. The total number of cells scored for each mutant is shown in *parentheses* above each *bar*. Results are presented as the means ± S.D. of at least three independent experiments.

shown). Second, the C809Y and R589M mutations, which inhibit KSR association with MEK (12, 13), also prevented the nuclear import of KSR (Fig. 4). Therefore, it is more likely that interaction with MEK enhances KSR nuclear export.

*KSR.NLS and KSR.T274V/S392A Bind to MEK*—To support the possibility that the re-localization of KSR.NLS and KSR.T274V/S392A from the nucleus to the cytoplasm upon coexpression with YFP-MEK1 was due to protein/protein interaction, co-immunoprecipitation experiments were performed. CFP-KSR.NLS, CFP-KSR.C809YNLS, and CFP-KSR.T274V/S392A were cotransfected with YFP-MEK1 into 293T cells. The cells were lysed and immunoprecipitated with anti-MEK antibody, and Western blotting was performed with monoclonal anti-GFP antibody, which also recognizes CFP and YFP (Fig. 9). The anti-GFP blot revealed that KSR.NLS and KSR.T274V/S392A co-immunoprecipitated with YFP-MEK1, consistent with the notion that cytoplasmic re-localization of KSR.NLS and KSR.T274V/S392A was mediated by direct interaction with MEK1. This conclusion is supported by the observation that KSR.C809YNLS did not co-precipitate with YFP-MEK1 and remained in the nucleus when coexpressed with YFP-MEK1 (Fig. 8).

*KSR Re-localizes MEK1.ΔNES to the Cytoplasm*—We examined whether the subcellular location of MEK1 alters its ability to affect the distribution of KSR in cells. MEK1 accumulates in the nucleus upon treatment with leptomycin B (31), suggesting that MEK1 shuttles in and out of the nucleus. An NES has been identified in the amino terminus (amino acids 32–44) of MEK1, and deletion of this region causes MEK1 to accumulate in the nucleus (31). Together with previous data showing that

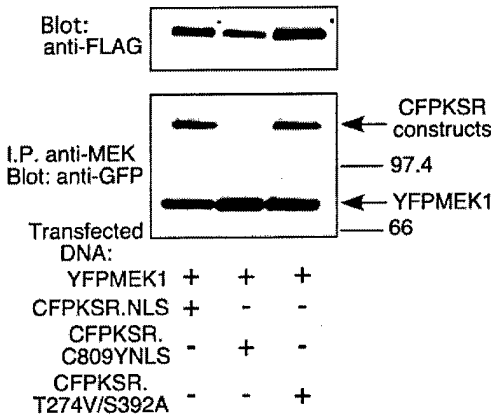


FIG. 9. **KSR.NLS and KSR.T274V/S392A bind to MEK.** CFP-KSR.NLS, CFP-KSR.C809YNLS, and CFP-KSR.T274V/S392A were transfected with YFP-MEK1 into 293T cells. The cells were lysed and immunoprecipitated (*I.P.*) with anti-MEK antibody, and Western blotting was performed with monoclonal anti-GFP antibody. The location of YFP-MEK1 and the CFP-KSR constructs is indicated. The *upper panel* shows the results from Western blotting performed with anti-FLAG antibody on a portion of the lysates to detect expression of the CFP-KSR constructs in the experiment.

mutations KSR.R589M and KSR.C809Y affect binding to MEK1 (12, 13), these data suggest a potential relationship between the nucleocytoplasmic transport of KSR and its ability to interact with MEK. To determine whether localizing MEK1 to the nucleus would cause redistribution of KSR, the NES in

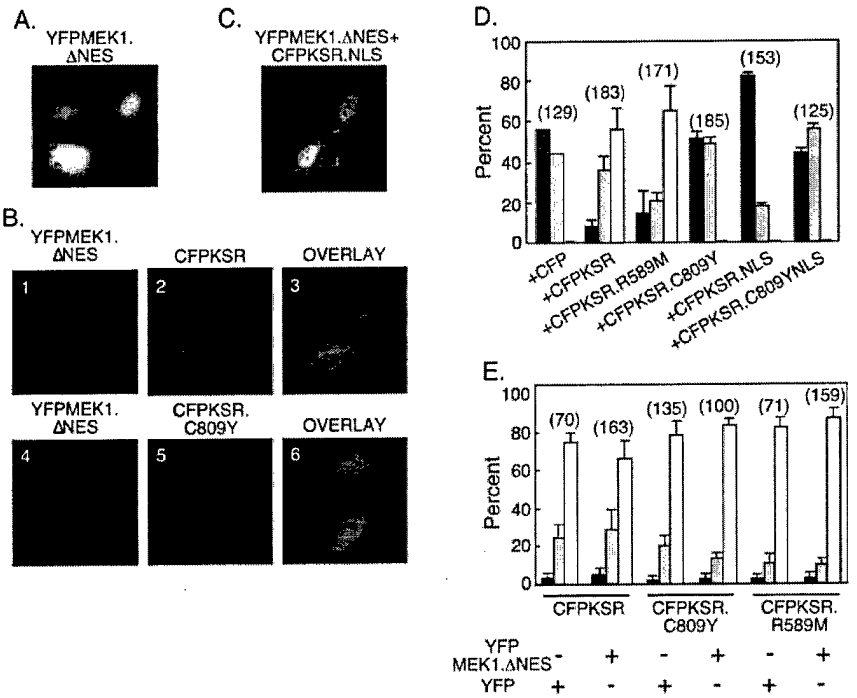


FIG. 10. **KSR re-localizes MEK1.ΔNES from the nucleus to the cytoplasm.** *A*, YFP-MEK1.ΔNES was expressed in REF-52 cells, and fluorescence microscopy was performed to determine its subcellular location. *B*, CFP-KSR and CFP-KSR.C809Y were coexpressed with YFP-MEK1.ΔNES in REF-52 cells, and fluorescence microscopy was performed to detect the location of the YFP and CFP constructs simultaneously. The *red* pseudocolor (*panels 1* and *4*) indicates the location of YFP-MEK1.ΔNES, and the *green* pseudocolor (*panels 2* and *5*) reveals the location of CFP-KSR and CFP-KSR.C809Y. *Panels 3* and *6* are the overlays of the YFP and CFP fluorescence. *C*, YFP-MEK1.ΔNES was coexpressed with CFP-KSR.NLS, and the location of YFP-MEK1.ΔNES was detected by fluorescence microscopy. *D*, YFP-MEK1.ΔNES was expressed with CFP or the indicated CFP-KSR constructs. The location of the indicated CFP-KSR constructs was detected by fluorescence microscopy, and cells were scored and grouped as nuclear (*dark gray bars*), nuclear and cytoplasmic (*light gray bars*), or cytoplasmic (*white bars*) as described in the legend to Fig. 8*C*. *E*, the indicated CFP-KSR constructs were expressed with YFP or YFP-MEK1.ΔNES. The location of YFP-MEK1.ΔNES was detected by fluorescence microscopy. Cells were grouped and scored as described for *D*. The total number of cells scored in *D* and *E* for each condition is shown in *parentheses* above each *bar*. Results in *D* and *E* are presented as the means ± S.D. of at least three independent experiments.

YFP-MEK1 was deleted (YFP-MEK1.ΔNES), and the deletion mutant was expressed in REF-52 cells. The ΔNES mutation dramatically shifted YFP-MEK to the nucleus (Fig. 10). Importantly, no cells were found to contain YFP-MEK1.ΔNES exclusively in the cytoplasm (Fig. 10*A*). When coexpressed with YFP-MEK1.ΔNES, the subcellular distribution of all CFP-KSR constructs did not significantly change (Fig. 10, *B* and *E*). However, CFP-KSR caused a redistribution of YFP-MEK1.ΔNES from the nucleus to the cytoplasm (Fig. 10, *B* and *D*). CFP-KSR.R589M had a similar effect on re-localizing YFP-MEK1.ΔNES from the nucleus to the cytoplasm (Fig. 10*D*), indicating that a KSR mutant with reduced binding to MEK1 is still sufficient to affect the localization of YFP-MEK1.ΔNES. However, coexpression of CFP-KSR.C809Y did not affect the localization of YFP-MEK1.ΔNES, suggesting that a direct association between KSR and MEK causes the redistribution of MEK1 to the cytoplasm.

Cytoplasmic forms of MEK1 redistributed KSR.NLS from the nucleus to the cytoplasm (Fig. 8). Similarly, coexpression of KSR re-localized MEK1.ΔNES from the nucleus to the cytoplasm (Fig. 10). These data raise the possibility that KSR and MEK interact preferentially in the cytoplasm. To determine whether KSR and MEK1 interact in the nucleus, YFP-MEK1.ΔNES and CFP-KSR.NLS were coexpressed in REF-52 cells. We found that coexpression of CFP-KSR.NLS enhanced the nuclear localization of YFP-MEK1.ΔNES, whereas coexpression of CFP-KSR.C809YNLS had no effect on YFP-MEK1.ΔNES localization (Fig. 10, *C* and *E*). These data suggest that KSR and MEK1 can also interact in the nucleus.

## DISCUSSION

The subcellular localization of components that compose signal transduction pathways has become an important focus in elucidating the mechanisms by which these pathways are regulated. KSR is an important regulator of the Raf/MEK/ERK kinase cascade (4–6, 8–10, 14, 16, 36). To gain insight into the mechanism by which KSR regulates ERK activation, we examined the subcellular localization of KSR. We found that KSR was localized to the cytoplasm of quiescent cells. The observation that a few cells expressing GFP-KSR had nuclear fluorescence led us to determine whether wild-type KSR undergoes nucleocytoplasmic shuttling. Treatment of REF-52 cells expressing GFP-KSR with leptomycin B, an inhibitor of Crm1-mediated nuclear export, caused GFP-KSR to accumulate in the nucleus of cells, suggesting that KSR is transported continuously in and out of the nucleus in quiescent cells.

Several phosphorylation sites have been identified on KSR (16, 33). Of the phosphorylation sites identified, mutation of $Thr^{274}$ and $Ser^{392}$ caused KSR to localize to the nucleus (Fig. 3). Phosphorylation of $Ser^{297}$ and $Ser^{392}$ regulates the interaction of KSR with 14-3-3 proteins (9, 16). Our observation that KSR.T274V/S392A was preferentially located in the nucleus implicates 14-3-3 as a regulator of KSR subcellular distribution. 14-3-3 functions in a similar manner to sequester the protein phosphatase Cdc25C (37), the forkhead transcription factor FHKRL1 (38), and Raf (39, 40). Combined mutation of $Ser^{297}$ and $Ser^{392}$ enhanced nuclear translocation of KSR (Fig. 3*B*); however, the nuclear shift was comparable to mutation of $Ser^{392}$ alone, suggesting that $Ser^{392}$ may be more important for

cytoplasmic retention of KSR through interaction with 14-3-3.

To determine whether $Thr^{274}$ and $Ser^{392}$ are the sole determinants of the cytoplasmic localization of KSR, we reintroduced $Thr^{274}$ and $Ser^{392}$ separately and in combination to the backbone of KSR.9mut. The observation that restoration of the $Thr^{274}$ and $Ser^{392}$ phosphorylation sites to the backbone of KSR.9mut did not completely prevent nuclear localization of KSR supports the possibility that other phosphorylation sites may regulate KSR subcellular localization. However, we cannot rule out the possibility that the combined mutation of the remaining seven phosphorylation sites may have a nonspecific effect on KSR subcellular localization. The mechanism through which $Thr^{274}$ synergizes with $Ser^{392}$ to regulate nuclear localization of KSR is not clear. $Thr^{274}$ may be phosphorylated by ERKs (16), but the subcellular location at which this phosphorylation occurs is not known. It is possible that phosphorylation of $Thr^{274}$ facilitates export of KSR from the nucleus. In this case, KSR.T274V/S392A may be localized to the nucleus because of increased nuclear import due to loss of 14-3-3 binding and decreased nuclear export due to loss of phosphorylation on $Thr^{274}$.

KSR is located in the cytoplasmic fraction and moves to the membrane upon stimulation with serum or platelet-derived growth factor (9, 41) or in the presence of activated Ras (8). In combination with the demonstration of KSR nuclear shuttling, these data suggest a mechanism similar to that affecting Ste5, a MAPK pathway scaffold in *Saccharomyces cerevisiae*. Ste5 continuously shuttles through the nucleus and translocates to the cell membrane upon pheromone stimulation (42). Ste5 proteins that do not cycle through the nucleus are unable to translocate to the cell membrane and inhibit activation of the MAPK pathway. Conversely, Ste5 with enhanced ability to cycle through the nucleus has an increased ability to localize to the cell membrane and to activate the pathway (42). It is intriguing to speculate that, like Ste5, KSR must cycle through the nucleus before translocating to the cell membrane upon mitogen stimulation.

Similar to KSR, MEK accumulates in the nucleus of quiescent cells upon treatment with leptomycin B (31), suggesting that MEK also shuttles continuously in and out of the nucleus. MEK contains an NES in its amino-terminal region that keeps MEK localized to the cytoplasm in quiescent cells (43, 44). Disruption of MEK subcellular localization may be the mechanism by which ectopic KSR inhibits ERK activation (7, 11, 14).

We hypothesize that KSR is a scaffold that regulates signaling through the Raf/MEK/ERK kinase cascade by regulating the subcellular distribution of MEK. KSR.C809Y does not bind MEK (12, 13), translocate to the nucleus (Fig. 8, *A* and *B*), or regulate ERK activity (Fig. 4*C*), suggesting a possible link between these different effects of KSR. We examined the role KSR may play in regulating MEK nuclear localization. We found that KSR constructs localized to the nucleus were relocalized to the cytoplasm when coexpressed with MEK1. Redistribution of KSR to the cytoplasm was also dependent on MEK binding. Thus, KSR.NLS and KSR.T274V/S392A, which both bound MEK1 (Fig. 9), redistribute from the nucleus to the cytoplasm upon coexpression with MEK1. Overexpression of MEK could indirectly affect KSR subcellular distribution. However, data that MEK coexpression does not alter the distribution of KSR.C809YNLS argue against this possibility because this construct did not bind MEK (Fig. 9). In addition, KSR.R589MNLS, a mutant with reduced MEK binding, showed an intermediate shift to the cytoplasm when coexpressed with MEK1. These observations suggest that a direct interaction between KSR and MEK affects the cytoplasmic distribution of KSR.

To determine whether altering the localization of MEK affects the location of KSR in REF-52 cells, we deleted the NES (amino acids 32–51) in MEK and fused YFP to the amino terminus (YFP-MEK.ΔNES). Greater than 50% of the cells had YFP-MEK.ΔNES preferentially located in the nucleus. Ectopic expression of any CFP-KSR construct able to bind MEK caused YFP-MEK.ΔNES to relocate to the cytoplasm. However, CFP-KSR.C809Y did not cause this re-localization. These studies indicate that interaction between KSR and MEK is critical in determining each protein's localization and suggest a potential role for KSR in the nuclear export of MEK. From these studies, it is unclear what role KSR may play in MEK export. One possibility is that KSR is exported with MEK. Although no NES has been identified, KSR does contain two regions that resemble leucine-rich NES motifs. The spacing of leucine/hydrophobic amino acids between amino acids 45 and 56 (**LIDISIGSLRGL**) and amino acids 827 and 837 (**LERLPKLNRRL**) resembles a consensus leucine-rich NES (45). Mutation of these regions may provide further insight into the role of KSR in MEK nucleocytoplasmic transport and signaling.

A popular model suggests that the intensity and duration of signaling through the Raf/MEK/ERK kinase cascade determine the nucleocytoplasmic distribution of ERKs and the subsequent response of a cell to the activation of this pathway (46). Ectopic expression of KSR at low levels has been reported to sustain ERK activation in epidermal growth factor-treated PC12 cells, with a coincident shift in cell responsiveness from proliferation to differentiation (13). Our observation that KSR translocates to the nucleus raises the intriguing possibility that it may affect the cellular response to activation of the MAPK pathway by affecting the subcellular distribution of key effectors within this pathway. If correct, identifying the regulators of KSR subcellular distribution may reveal important modifiers of MAPK signaling.

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# Susceptibility to Estrogen-Induced Mammary Cancer Segregates as an Incompletely Dominant Phenotype in Reciprocal Crosses between the ACI and Copenhagen Rat Strains

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**Estrogens have been inextricably linked to the etiology of breast cancer. We have demonstrated that the female ACI rat exhibits a unique propensity to develop mammary cancers when treated continuously with physiological levels of 17β-estradiol (E2). The E2-induced mammary cancers are estrogen dependent and exhibit genomic instability. In contrast, the genetically related Copenhagen (COP) rat strain is relatively resistant to E2-induced mammary cancers. In this study we evaluated susceptibility to E2-induced mammary cancers in first filial ($F_1$), second filial ($F_2$), and backcross (BC) progeny generated from reciprocal intercrosses between the ACI and COP strains. $F_1$ progeny resembled the parental ACI strain with respect to incidence of E2-induced mammary cancers. However, latency was significantly prolonged in the $F_1$ populations. These data indicate that susceptibility behaves as an incompletely dominant phenotype in these crosses. Analysis of phenotypes exhibited by the $F_1$, $F_2$, and BC populations suggests that mammary cancer susceptibility is modified by one or two genetic loci in the reciprocal intercrosses between the ACI and COP strains. Susceptibility to E2-induced mammary cancers did not correlate with E2-induced pituitary growth in the genetically diverse $F_2$ and BC populations, suggesting that the genetic bases for susceptibility to E2-induced mammary cancers differ from those for E2-induced lactotroph hyperplasia. (*Endocrinology* 142: 5124–5130, 2001)**

NUMEROUS EPIDEMIOLOGICAL and laboratory studies indicate that estrogen plays a central role in the etiology of breast cancer (1, 2). Moreover, a recent prevention study demonstrated that the antiestrogen tamoxifen reduces by approximately one half the incidence of breast cancer in a population of women at elevated risk for this disease (3). Although these studies clearly indicate that ER-mediated pathways contribute to breast cancer, these pathways have not been precisely defined.

We have demonstrated that the female of the inbred ACI rat strain exhibits a unique propensity to develop mammary cancers when treated with E2; continuous treatment with E2 induced mammary cancers at an incidence of 100% and a median latency of 143 d (4, 5). In contrast, the female of the Copenhagen (COP) strain, an inbred strain closely related genetically to the ACI strain, is relatively resistant to E2-induced mammary cancers (5, 6). Interestingly, both ACI and COP rats are relatively resistant to mammary cancers induced by dimethylbenz[*a*]anthracene (DMBA) (7–10) or *N*-methyl-*N*-nitrosourea (MNU) (7, 10–12). These data suggest that the molecular mechanisms underlying development of E2-induced mammary cancers differ from those for DMBA- or MNU-induced mammary cancers. We have recently demonstrated that the mammary epithelium of the female ACI rat proliferates in response to administered E2 to a greater extent than does the mammary epithelium of the female COP rat, as evidenced by morphometric analysis of the epithelium to adipose tissue ratio and quantification of S phase indexes (13). Together these data suggest that the differing susceptibilities of ACI and COP rats to E2-induced mammary cancers are genetically conferred and result at least in part from differences in the extent to which the mammary epithelia of these two rat strains proliferate in response to E2. A goal of this laboratory is to define the molecular bases for these strain differences in the hope that this information may provide novel insights into the mechanisms through which estrogen contributes to breast cancer etiology in humans.

As the first step in defining the genetic bases of the unique susceptibility of the ACI rat to 17β-estradiol (E2)-induced mammary cancers, we examined how susceptibility to these cancers segregates in reciprocal crosses between the genetically related ACI and COP rat strains. The data presented herein indicate that susceptibility to E2-induced mammary cancer segregates as an incompletely dominant trait in these reciprocal intercrosses.

Abbreviations: BC, Backcross; BCa, (ACI × $F_{1a}$)BC; BCb, (ACI × $F_{1b}$)BC; BN, Brown Norway; COP, Copenhagen; DMBA, dimethylbenz[*a*]anthracene; $F_1$, first filial; $F_2$, second filial; MNU, *N*-methyl-*N*-nitrosourea.

## Materials and Methods

### *Care and treatment of animals*

The institutional animal care and use committee of the University of Nebraska Medical Center approved all procedures involving live animals. ACI rats were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). COP rats were obtained from the NCI breeding program.

Animals were housed in the Eppley Institute barrier animal facility under controlled temperature, humidity, and lighting conditions. This facility is accredited by the American Association for Accreditation of Laboratory Animal Care and is operated in accordance with the standards outlined in Guide for the Care and Use of Laboratory Animals. The rats were housed in polycarbonate cages on corncob bedding and were allowed continuous access to standard laboratory chow (Harlan Teklad, Madison, WI) and water.

Reciprocal ACI female × COP male (cross A) and COP female × ACI male (cross B) intercrosses were performed to generate (ACI × COP)$F_1$ ($F_{1a}$) and (COP × ACI)$F_1$ ($F_{1b}$) progeny, respectively. $F_1$ siblings from each cross were mated to generate $F_{2a}$ and $F_{2b}$ progeny. $F_{1a}$ and $F_{1b}$ males were mated to ACI females to generate backcross (BC) a and BCb progeny, respectively. All pups were weaned at 21 ± 2 d of age. Treatment with E2 was initiated when the rats were 63 ± 4 d of age. SILASTIC brand implants (Dow Corning Corp., Midland, MI) containing 27.5 mg crystalline E2 (Sigma, St. Louis, MO) were prepared and inserted sc in the interscapular region as described previously (4). Beginning approximately 5 wk after initiation of E2 treatment, each animal was examined twice weekly for the presence of palpable mammary tumors. An animal was killed by decapitation when its largest mammary tumor reached 1.5–2.0 cm in diameter or when it exhibited other treatment-related pathology. The location and size of each macroscopic mammary tumor were noted at necropsy. Mammary tissues, both grossly normal and tumors, were collected and evaluated histologically as described previously (4, 13). The pituitary gland was removed and weighed as an indicator of E2-induced lactotroph hyperplasia; pituitary weight correlates with lactotroph number and circulating PRL level (14).

### *Statistical analysis of data*

Latency was scored to the appearance of the first palpable mammary tumor. Phenotypic differences between different genetic populations were assessed by the log-rank test and Wilcoxon rank-sum/Mann-Whitney U test. Where appropriate, differences between population means were evaluated using two-tailed $t$ test. $P \leq 0.05$ was considered statistically significant.

## Results

### *ACI and COP strains differ markedly in susceptibility to estrogen-induced mammary cancers*

Treatment with E2 induced rapid development of mammary carcinomas in female ACI rats (Fig. 1A). The first mammary tumor in the treated ACI population was observed after 88 d of E2 treatment, and 100% of the population at risk exhibited at least 1 palpable mammary tumor within 197 d of initiation of treatment. Three of 34 E2-treated ACI rats were killed before the appearance of palpable mammary tumors due to treatment-associated morbidities after 144, 165, and 175 d of treatment, resulting in an overall incidence of 91% in this population. Within the 31 tumor-positive ACI rats, the median and mean latencies to the appearance of the first palpable tumor were 140 and 141 d, respectively (Table 1). In contrast, the first mammary tumor to appear in the population of E2-treated COP rats was observed after 188 d of E2 treatment, and 44% of the population at risk, 35% (7 of 20) of the total COP population, ultimately developed mammary cancers (Fig. 1B). The median and mean latencies within the tumor-positive COP population were 195 and 208 d, respectively. Log-rank analyses indicated that the ACI and COP rat strains differed significantly with respect to latency to appearance of the first palpable mammary cancer ($P < 10^{-6}$).

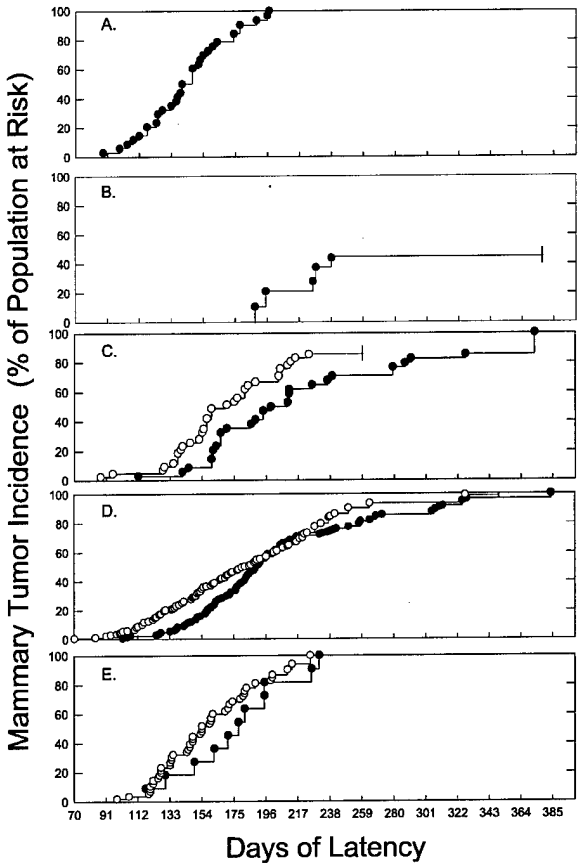


FIG. 1. Susceptibility to E2-induced mammary cancer segregates as an incomplete dominant trait in reciprocal ACI × COP intercrosses. Ovary-intact, virgin females rats were treated with E2 beginning at 9 wk of age and were monitored twice weekly for the presence of palpable mammary cancers. Each *data point* represents the time point at which an animal in the population at risk exhibited its first palpable mammary cancer. A, ACI; B, COP; C, ●, $F_{1a}$; ○, $F_{1b}$. D, ●, $F_{2a}$; ○, $F_{2b}$. E, ●, BCa; ○, BCb. Mammary cancers did not develop in untreated, ovary-intact ACI, COP, $F_1$, $F_2$, or BC rats over the time course examined in this study.

### *Latency to appearance of first estrogen-induced mammary cancer behaves as an incompletely dominant genetic trait*

The first mammary carcinoma in the (ACI × COP)$F_1$ ($F_{1a}$) population was observed after 112 d of E2 treatment, and 100% of the $F_{1a}$ population at risk, 88% (30 of 34) of the total $F_{1a}$ population, developed mammary cancer within 373 d of initiation of E2 treatment (Fig. 1C). Median and mean latencies in the tumor-positive $F_{1a}$ population were 194 and 207 d, respectively (Table 1). In the (COP × ACI)$F_1$ ($F_{1b}$) population, the first mammary tumor was observed after 87 d of E2 treatment, and 85% of the $F_{1b}$ population at risk, 81% (35 of 43) of the total $F_{1b}$ population, were tumor positive within 259 d of initiation of E2 treatment (Fig. 1C). Median and mean latencies to the appearance of the first palpable mammary tumor in the $F_{1b}$ population were 157 and 163 d, respectively (Table 1). Comparison of the E2-treated $F_{1a}$ and $F_{1b}$ populations by the log-rank test indicated that they differed significantly ($P = 0.02$). Although the incidence of E2-induced mammary cancers in the $F_{1a}$ and $F_{1b}$ populations approached

**TABLE 1.** Phenotypes of ACI and COP rat strains in comparison to $F_1$, $F_2$, and BC progeny from reciprocal ACI × COP intercrosses

| Strain | Population size | Incidence (%)[a] | Median latency (d)[b] | Mean latency (d)[b] | Tumor no.[c] | Length of treatment (d)[d] |
|---|---|---|---|---|---|---|
| ACI | 34[e] | 100/91.2 | 140 | 141 ± 27 (n = 31) | 3.9 ± 3.5 (0–18) | 171 ± 24 |
| COP | 20 | 43.8/35.0 | 195 | 208 ± 20 (n = 7) | 0.4 ± 0.6 (0–2) | 271 ± 59 |
| F1a | 34 | 100/88.2 | 194 | 207 ± 59 (n = 30) | 2.6 ± 2.0 (0–9) | 267 ± 55 |
| F1b | 43 | 85.4/81.4 | 157 | 163 ± 32 (n = 35) | 1.8 ± 1.3 (0–5) | 224 ± 23 |
| F1 | 77 | 100/84.4 | 170 | 184 ± 50 (n = 65) | 2.1 ± 1.7 (0–9) | 243 ± 46 |
| F2a | 134 | 99.1/85.8 | 184 | 193 ± 50 (n = 115) | 2.3 ± 1.9 (0–11) | 240 ± 57 |
| F2b | 172 | 99.1/60.5 | 151 | 157 ± 44 (n = 104) | 1.2 ± 1.4 (0–8) | 212 ± 41 |
| F2 | 306 | 99.6/71.6 | 171 | 176 ± 51 (n = 219) | 1.7 ± 1.7 (0–11) | 224 ± 51 |
| BCa | 11 | 100/100 | 178 | 176 ± 34 (n = 11) | 3.3 ± 2.1 (1–7) | 219 ± 42 |
| BCb | 57 | 100/86.0 | 148 | 154 ± 30 (n = 49) | 2.7 ± 2.7 (0–17) | 199 ± 32 |
| BC | 68 | 100/88.2 | 160 | 158 ± 32 (n = 60) | 2.8 ± 2.6 (0–17) | 203 ± 34 |

[a] Incidence in population at risk/incidence in total population.
[b] Median and mean (±SD) latency determined for tumor-positive animals in E2-treated population.
[c] Average tumor number per rat (±SD; range indicated in *parentheses*) determined at time of death for total E2-treated population.
[d] Mean duration (±SD) of E2 treatment.
[e] A single animal in a population of 35 died after 26 d of E2 treatment and was excluded from analysis.

that in the ACI parental strain, the latencies in both $F_1$ populations were significantly prolonged relative to that of the ACI population ($P \leq 10^{-6}$, ACI *vs.* $F_{1a}$; $P = 1.33 \times 10^{-4}$, ACI *vs.* $F_{1b}$). Both $F_1$ populations also differed significantly from the COP population ($P = 1.6 \times 10^{-3}$, COP *vs.* $F_{1a}$; $P = 2.9 \times 10^{-5}$, COP *vs.* $F_{1b}$). Because the two different $F_1$ populations exhibited a phenotype intermediate compared to those of the ACI and COP rat strains, we conclude that susceptibility to E2-induced mammary cancers is inherited as an incompletely dominant trait in these reciprocal intercrosses between the ACI and COP rat strains.

The ability of E2 to induce mammary carcinomas was evaluated in 134 ($F_{1a} \times F_{1a}$)$F_2$ ($F_{2a}$) and 172 ($F_{1b} \times F_{1b}$)F2 ($F_{2b}$) progeny (Fig. 1D). Mammary cancers were first observed in the E2-treated $F_{2a}$ and $F_{2b}$ populations following 102 and 70 d of treatment, respectively, and the final incidence in the $F_{2a}$ and $F_{2b}$ populations at risk exceeded 99% (Fig. 1D). The incidences of E2-induced mammary cancers in the total $F_{2a}$ and $F_{2b}$ populations were 86% (115 of 134) and 61% (104 of 172), respectively (Table 1). Median and mean latencies in the tumor-positive $F_{2a}$ populations were 184 and 193 d, respectively. In the tumor-positive $F_{2b}$ populations, median and mean latencies were 151 and 157 d, respectively. Log-rank analysis indicated that the two $F_2$ populations did not differ significantly from one another with respect to latency to the appearance of the first palpable mammary cancer ($P = 0.80$). The combined $F_2$ population differed significantly from the ACI ($P < 10^{-6}$) as well as the COP ($P = 1.87 \times 10^{-4}$) populations and exhibited a prolonged latency relative to the $F_{1b}$ ($P = 0.041$), but not the $F_{1a}$ or combined $F_1$, populations.

Mammary cancers in the (ACI × $F_{1a}$)BC (BCa) and (ACI × $F_{1b}$)BC (BCb) populations were first observed 117 and 98 d, respectively, after initiation of E2 treatment (Fig. 1E). One hundred percent of the BCa population at risk and the total BCa population developed at least one palpable mammary cancer within 231 d of initiation of E2 treatment (Table 1). Similarly, 100% of the BCb population at risk, 86% of the total BCb population, was tumor positive by 225 d after initiation of treatment. Log-rank analyses indicated that these two BC populations did not differ significantly from one another ($P = 0.252$). Mammary tumor development in the combined BC population was significantly delayed compared with that in the ACI population ($P = 6.03 \times 10^{-3}$), whereas tumors appeared more rapidly in the combined BC population than in the COP ($P < 10^{-6}$), combined $F_1$ ($P = 2.36 \times 10^{-4}$), or combined $F_2$ ($P < 10^{-6}$) populations.

**TABLE 2.** Mammary cancer incidence after 175 d of E2 treatment

| Population | Observed tumor positive (%) | Predicted tumor positive (%)[a] |
|---|---|---|
| ACI | 84.4 | |
| COP | 0.0 | |
| $F_1$ | 45.5 | 42.2 (>0.50) |
| $F_2$ | 41.1 | 42.2 (>0.70) |
| BC | 64.6 | 63.3 (>0.70) |

[a] Predicted by model where one gene, with ACI and COP alleles acting codominantly, determines susceptibility to E2-induced mammary cancers. Values in *parentheses* indicate the $\chi^2$ probability. Predicted phenotypes: $F_1$ = (observed ACI phenotype + observed COP phenotype)/2; $F_2$ = (observed ACI phenotype + (2 × predicted $F_1$ phenotype) + observed COP phenotype)/4; BC = (observed ACI phenotype + predicted $F_1$ phenotype)/2.

Presented in Table 2 are the percentages of each population exhibiting one or more mammary cancers after 175 d of E2 treatment, the time point that most clearly demarcates the phenotypic differences between the treated ACI and COP popu-

lations. At this time point, 84% of the E2-treated ACI rats exhibited mammary cancer in contrast to 0% for the COP population. The percentages of rats in the E2-treated $F_1$, $F_2$, and BC populations were 45%, 41%, and 65%, respectively. These data closely approximate the fractions of these populations predicted to be tumor positive by a genetic model in which one gene, with ACI and COP alleles acting codominantly, determines susceptibility to E2-induced mammary cancers. However, these data are also consistent with a model in which two independently segregating genes determine susceptibility.

*Genetically conferred susceptibility to estrogen-induced mammary cancers is reflected in tumor burden*

Tumor number in the E2-treated ACI population ranged from 0–18 and averaged 3.9 mammary tumors/rat at the time the animals were killed (Table 1). Twenty-seven of the 31 tumor-positive ACI rats (87%) exhibited multiple mammary tumors at necropsy. By contrast, tumor number in the E2-treated COP population ranged from 0–2, and averaged 0.4 tumors/animal at the time the animals were killed. Only 1 of 7 (14%) tumor-positive COP rats exhibited more than 1 tumor at necropsy. Tumor number at necropsy in the E2-treated ACI population differed significantly ($P = 0.006$) from that in the treated COP population. The average lengths of E2 treatment in the ACI and COP populations were 171 and 271 d, respectively.

The E2-treated $F_{1a}$ population exhibited, on the average, 2.6 mammary tumors/rat at the time of death, whereas the $F_{2b}$ population exhibited, on the average, 1.8 tumors/rat, a difference that was statistically significant ($P = 0.028$; Table 1). Tumor number in the $F_{1a}$ population did not differ significantly from that in the ACI population ($P = 0.077$). However, tumor number in the $F_{1b}$ population was significantly lower ($P = 0.0003$) than that in the E2-treated ACI population. Both the $F_{1a}$ and $F_{1b}$ populations exhibited significantly more mammary tumors at the time of death than did the E2-treated COP rats ($P \leq 0.0001$ for both comparisons). Tumor number in the $F_{2a}$ population averaged 2.3 tumors/rat, which was significantly greater ($P \leq 0.01$) than the average of 1.2 tumors/rat observed in the $F_{2b}$ population. The average numbers of tumors observed at the time of death in the $F_{2a}$ and $F_{2b}$ populations were significantly less than that observed in the ACI population and significantly greater than that observed in the COP population. The BCa and BCb populations exhibited an average of 3.3 and 2.7 mammary cancers/rat, respectively, at the time of death (Table 1), a difference that was not statistically significant. The average number of mammary tumors observed in the E2-treated BCa and BCb populations did not differ significantly from that observed in the treated ACI population, but was significantly greater ($P \leq 0.01$ for both comparisons) than that observed in the treated COP population. It is noteworthy that the $F_{1a}$, $F_{2a}$, and BCa progeny generated from the ACI × COP cross survived longer, on the average, than the $F_{1b}$, $F_{2b}$, and BCb progeny from the COP × ACI cross. It is possible that the longer duration of E2 treatment in the progeny from the ACI × COP cross contributed to the greater mammary tumor burden observed in these populations relative to that observed in the progeny from the COP × ACI cross.

*Histological features of estrogen-induced mammary cancers*

The mammary cancers induced by E2 in ACI and COP rats and their derived $F_1$, $F_2$, and BC progeny were adenocarcinomas of the comedo, papillary, or cribriform types. Invasive features were observed in a subset of the cancers. The histological appearance of the tumors was similar among the different genetic groups.

*Effects of estrogen treatment on pituitary weight*

Pituitary weight was measured to evaluate the possible association between E2-induced lactotroph hyperplasia and susceptibility to E2-induced mammary cancers. Pituitary weight in untreated, ovary-intact, female rats averaged 11.4 mg, and continuous treatment with E2 increased pituitary weight 5.3- to 17.2-fold in the different genetic groups (Fig. 2). Although pituitary weight at necropsy was increased to a similar extent in the E2-treated ACI and COP populations,

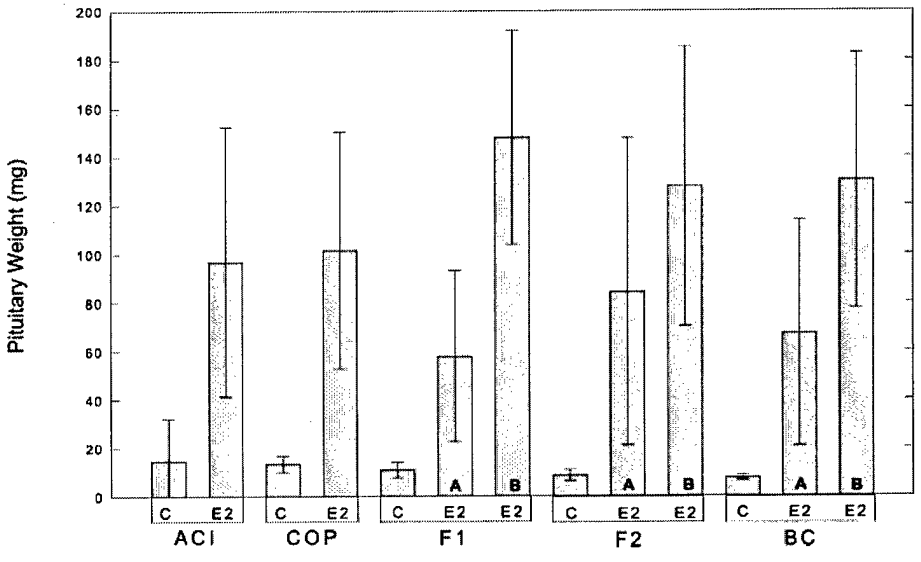


FIG. 2. Continuous treatment with E2 induces pituitary growth. Ovary-intact, virgin female rats were treated with E2 beginning at 9 wk of age as described in Fig. 1 and *Materials and Methods*. Pituitary weight was measured immediately after the death of each rat. Each *bar* represents the mean pituitary weight ± SD. C, Control; E2, treated continuously with E2; A, progeny generated in an ACI female × COP male cross; B, progeny generated in a COP female × ACI male cross.

the COP population was treated an average of 100 d longer than the ACI population. These data are consistent with our previous report that the ACI rat strain is more sensitive to the pituitary growth-inducing actions of estrogen than the COP rat strain (14). Interestingly, the stimulatory effect of E2 on pituitary growth was substantially less in the $F_{1a}$, $F_{2a}$, and BCa females derived from the ACI × COP cross than in the $F_{1b}$, $F_{2b}$, and BCb progeny derived from the COP × ACI cross (Fig. 2). No association was apparent between pituitary weight and susceptibility to E2-induced mammary cancers in the $F_2$ and BC progeny from the reciprocal crosses between the ACI and COP rat strains. In the E2-treated $F_2$ population, pituitary weights in those animals exhibiting a palpable mammary cancer before 175 d of treatment were, on the average, 16% less than those in animals exhibiting their first palpable mammary cancers after 175 d and 46% less than those in animals that did not develop mammary cancers (Table 3). In the E2-treated BC population, pituitary weights were, on the average, similar in each of the three phenotypic classes of animals. Pituitary weights in the $F_2$ and BC animals are plotted as a function of duration of E2 treatment and mammary cancer phenotype in Figs. 3 and 4, respectively. These data clearly illustrate the high degree of variability in pituitary weights observed in the E2-treated $F_2$ and BC populations and the lack of correlation between pituitary weight and susceptibility to E2-induced mammary cancers.

## Discussion

The data presented herein indicate that the unique propensity of the female ACI rat to develop mammary cancers when treated with E2 behaves as an incompletely dominant trait in reciprocal genetic crosses with the resistant COP strain. Although the $F_1$ progeny resembled the parental ACI strain with respect to incidence of E2-induced mammary cancers, latency in the $F_1$ progeny was significantly prolonged, and tumor burden was significantly diminished relative to that observed for the female ACI rats. These data are in marked contrast to those from previous studies, which demonstrated that the resistant phenotype of the COP strain to DMBA- and MNU-induced mammary cancers was dominant relative to the more highly susceptible phenotype of several other inbred rat strains (7–10, 15, 16). Most noteworthy in this regard is the study by Isaacs (8) in which female ACI, (ACI × COP)$F_1$, and COP rats developed 0.3, 0.05, and 0.0 mammary cancers, respectively, after treatment with DMBA, indicating dominant inheritance of the resistant COP phenotype in that cross to the ACI strain. Together these studies indicate that the genetic bases of susceptibility to E2-induced mammary cancers differ from those for susceptibility to DMBA- and MNU-induced mammary cancers. We

**TABLE 3.** Pituitary weight as a function of susceptibility to E2-induced mammary cancers

| Relative susceptibility | Pituitary wt (mg)[a] | |
|---|---|---|
| | $F_2$ progeny | BC progeny |
| Tumor positive ≤175 d | 83.5 ± 56.3 | 120.9 ± 63.0 |
| Tumor positive ≥175 d | 99.9 ± 64.5 | 118.2 ± 48.1 |
| Tumor negative | 154.2 ± 47.3 | 117.2 ± 34.0 |

[a] Mean pituitary weight ± SD.

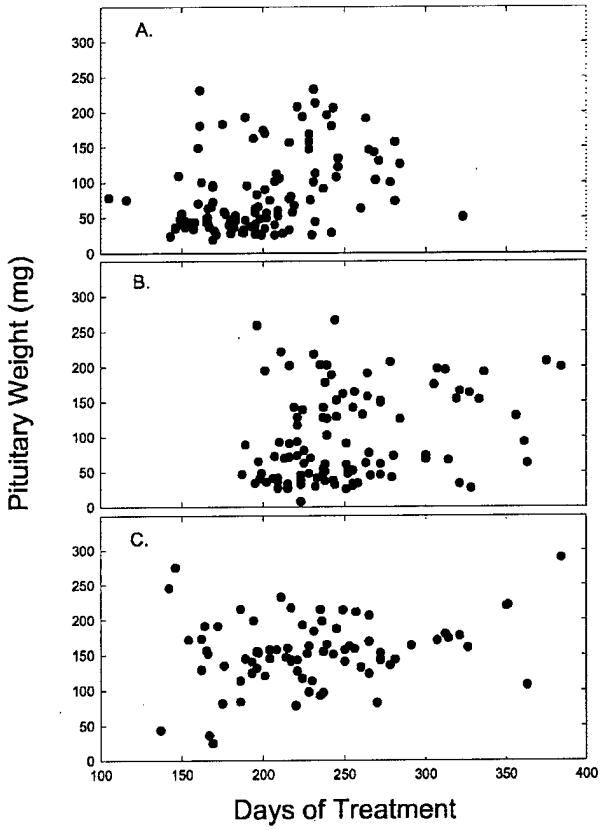


FIG. 3. Pituitary weights in $F_2$ rats defined phenotypically with respect to susceptibility to E2-induced mammary cancer. Each *data point* represents the pituitary weight for one $F_2$ rat treated with E2 for the indicated period. A, Pituitary weights in $F_2$ rats exhibiting one or more palpable mammary cancers within 175 d after initiation of E2 treatment. B, Pituitary weights in $F_2$ rats exhibiting one or more palpable mammary cancers after 175 d or more of E2 treatment. C, Pituitary weights in $F_2$ rats that were free of palpable mammary cancers at the time of death.

subsequently generated data similar to those presented herein in an intercross between ACI females and Brown Norway (BN) males; the BN strain is unrelated to ACI and exhibits resistance to E2-induced mammary cancers (Tochacek, M., *et al.*, unpublished data). Together these data strongly suggest that the incompletely dominant inheritance of susceptibility to E2-induced mammary cancers in the ACI × COP intercrosses is not simply due to the genetic relatedness of the ACI and COP strains. Because of the recognized role of estrogen in the genesis of breast cancer in humans, we anticipate that comparative studies of E2-induced mammary carcinogenesis in the highly susceptible ACI strain and the resistant COP and BN strains will provide novel insights into the mechanisms through which estrogen contributes to breast cancer development.

The observation that susceptibility to E2-induced mammary cancers in reciprocal ACI × COP intercrosses is inherited as an incompletely dominant phenotype is consistent with multiple genetic models. In the first model the ACI allele of a single gene, inherited through the germ line, would confer susceptibility in $F_1$ progeny. Somatic loss of the COP allele of this putative tumor suppressor gene would be re-

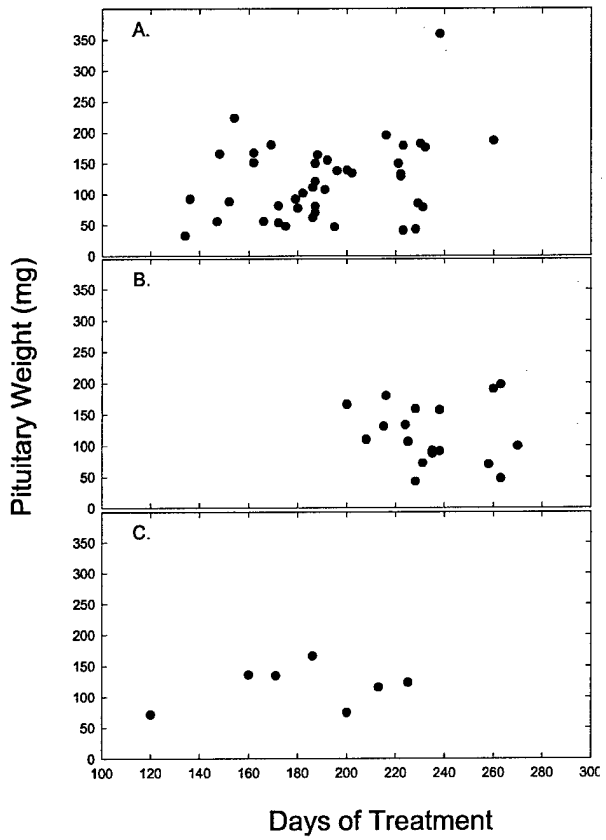


FIG. 4. Pituitary weights in BC rats defined phenotypically with respect to susceptibility to E2-induced mammary cancer. Each *data point* represents the pituitary weight for one BC rat treated with E2 for the indicated period. A, Pituitary weights in BC rats exhibiting one or more palpable mammary cancers within 175 d after initiation of E2 treatment. B, Pituitary weights in BC rats exhibiting one or more palpable mammary cancers after 175 d or more of E2 treatment. C, Pituitary weights in BC rats that were free of palpable mammary cancers at the time of death.

quired for mammary tumors to develop in $F_1$ animals. Consequently, latency to tumor appearance would be prolonged, and tumor burden would be reduced in $F_1$ females relative to that observed in the parental ACI strain. In this model genomic instability could contribute to COP allele loss and mammary cancer development. We demonstrated that the mammary cancers induced in ACI rats by E2 commonly exhibit aneuploidy (13). Moreover, unpublished data from our laboratory indicate widespread allelic imbalances consistent with LOH in mammary cancers induced in (ACI × COP)$F_1$ progeny by E2 (17). Thus, genetic instability is a common feature of E2-induced mammary cancers, and this could contribute to mammary carcinogenesis in this model. The phenotypic data presented herein are also consistent with a model in which two independently segregating genes confer susceptibility to E2-induced mammary cancers. Using the formula: $n = (\mu_{F1} - \mu_{ACI})^2/4\sigma_G^2$ (18), where n represents the number of loci modifying a specific trait, $\mu_{F1}$ and $\mu_{ACI}$ represent the phenotypic means of the $F_1$ and ACI populations, respectively, and $\sigma_G^2$ represents the genetic variance of the backcross population, it is estimated that no more than two independently segregating genes confer susceptibility to E2-induced mammary cancers in the ACI × COP intercrosses. Using the phenotypically defined $F_2$ and BC populations described herein, we have mapped to rat chromosome 5 a locus, *Emca1*, that modifies susceptibility to E2-induced mammary cancers in the reciprocal ACI × COP intercrosses (19) (Tochacek, M., T. M. Reindl, C. R. Murrin, E. A. VanderWoude, and J. D. Shull, manuscript in preparation).

For the most part, the mammary cancer profiles generated in the two reciprocal crosses between the ACI and COP rat strains were very similar. The $F_{1a}$ population from the ACI × COP cross exhibited a significantly prolonged latency relative to the $F_{1b}$ population from the COP × ACI cross. However, the $F_2$ and BC populations from the two crosses did not differ significantly from one another. The numbers of mammary tumors observed at necropsy were also somewhat higher in the $F_1$, $F_2$, and BC populations from the ACI × COP cross relative to the numbers observed in the corresponding populations from the COP × ACI cross. Therefore, we cannot exclude at this time the possibility that latency and/or tumor burden might be modified by either an X-linked or imprinted autosomal locus.

It has often been suggested that mammary cancers that develop in rats that are treated chronically with estrogen are secondary to the development of PRL-producing pituitary tumors and associated hyperprolactinemia (20–22). Pituitary weight in estrogen-treated rats correlates with the absolute lactotroph number (23) as well as with the circulating PRL level (14, 23). Although the ACI and COP rat strains differ markedly in susceptibility to E2-induced mammary cancers, both strains exhibit significant pituitary growth and hyperprolactinemia in response to administered estrogen (4, 6, 14). In the present study each of the E2-treated ACI, COP, $F_1$, $F_2$, and BC animals exhibited significantly increased pituitary weight relative to untreated female rats of the same genetic background. Moreover, in the genetically diverse $F_2$ and BC populations, latency to appearance of mammary carcinoma did not correlate with pituitary weight at the time of death. Finally, we have demonstrated that ovariectomy markedly inhibits E2-induced mammary carcinogenesis in the ACI rat without inhibiting pituitary growth and associated hyperprolactinemia (4). Together, these data indicate that E2-induced pituitary growth and associated hyperprolactinemia are insufficient for mammary cancer development. This, in turn, implies that the mammary cancers that develop in response to continuous E2 treatment are not simply the consequence of pituitary tumor-associated hyperprolactinemia. We have recently mapped four quantitative trait loci in ACI × COP crosses that modify the pituitary growth response to administered estrogen (Strecker, T. E., T. J. Spady, A. Kaufman, F. Chen, and J. D. Shull, manuscript in preparation). These quantitative trait loci are distinct from the *Emca1* modifier of susceptibility to E2-induced mammary cancers identified by us in similar crosses, indicating that the tumor-inducing actions of estrogen in the mammary gland are genetically dissociable from those in the pituitary gland.

The ACI rat appears by numerous criteria to represent a unique and physiologically relevant animal model for study of the molecular and hormonal etiology of breast cancer.

Mammary tumors develop rapidly and at high incidence in response to E2. The cancers are estrogen dependent and, like most breast cancers in humans, exhibit genomic instability (13). The data presented herein indicate that this unique susceptibility of the ACI rat to E2-induced mammary cancers segregates as an incompletely dominant phenotype in crosses to the COP rat strain, which is considered to represent the paradigm of genetically conferred resistance to experimental mammary cancer. Further elucidation of the genetic bases of susceptibility to E2-induced mammary cancers in this rat model should significantly enhance our understanding of the mechanisms through which estrogens contribute to breast cancer etiology and provide insights into the genetic epidemiology of breast cancer.

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